PCT/GB 2004 / 0 0 5 0 0 6

The Patent Office

INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

REC'D 17 JAN 2005
WIPO PCT

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed R. Mahoney

Dated 4 January 2005

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Patents Form 1/77

THE PATENT OFFICE
JG
26 NOV 2003
RECEIVED BY FAX

The Patent Office

1/77

Patents Act 1977
(Rule 16)

The Patent Office

Cardiff Road
Newport
South Wales
NP10 8QQ

26NOV03 E855198-1 D02884
P01/7700 0.00-0327499.0

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

1.	Your reference	P36104-/CMU/MCM
2.	Patent application number *(The Patent Office will fill in this part)*	0327499.0
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	The Queen's University of Belfast University Road Belfast BT7 1NN
	Patents ADP number *(if you know it)*	8103517001
	If the applicant is a corporate body, give the country/state of its incorporation	United Kingdom
4.	Title of the invention	"Cancer Treatment"
5.	Name of your agent *(if you have one)*	Murgitroyd & Company
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	Scotland House 165-169 Scotland Street Glasgow G5 8PL
	Patents ADP number *(if you know it)*	~~1198013~~ 1198015
6.	If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number	Country / Priority application number *(if you know it)* / Date of filing *(day / month / year)*
7.	If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application / Date of filing *(day / month / year)*
8.	Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:* *a) any applicant named in part 3 is not an inventor, or* *b) there is an inventor who is not named as an applicant, or* *c) any named applicant is a corporate body.* *See note (d))*	Yes

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	68
Claim(s)	6
Abstract	-
Drawing(s)	18 only

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*

Request for preliminary examination and search *(Patents Form 9/77)*

Request for substantive examination *(Patents Form 10/77)*

Any other documents *(please specify)*

11. I/We request the grant of a patent on the basis of this application.

Signature Murgitroyd & Co

Murgitroyd & Company

Date 26 November 2003

12. Name and daytime telephone number of person to contact in the United Kingdom MALCOLM MAIN 0141 307 8400

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) *If you need help to fill in this form or you have any questions, please contact the Patent Office on 08459 500505.*

b) *Write your answers in capital letters using black ink or you may type them.*

c) *If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.*

d) *If you have answered 'Yes' Patents Form 7/77 will need to be filed.*

e) *Once you have filled in the form you must remember to sign and date it.*

f) *For details of the fee and ways to pay please contact the Patent Office.*

Cancer Treatment

Field of the Invention

The present invention relates to cancer treatment. In particular, it relates to assays and methods of determining susceptibility to resistance to anti-cancer drugs such as fluoropyrimidines, and methods and compositions for treatment of cancer.

Background to the Invention

5-FU[4] is widely used in the treatment of a range of cancers including colorectal, breast and cancers of the aerodigestive tract. The mechanism of cytotoxicity of 5-FU has been ascribed to the misincorporation of fluoronucleotides into RNA and DNA and to the inhibition of the nucleotide synthetic enzyme thymidylate synthase (TS) (Longley et al., 2003). TS catalyses the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) with 5,10-methylene

tetrahydrofolate (CH2THF) as the methyl donor. This reaction provides the sole intracellular source of thymidylate, which is essential for DNA synthesis and repair. The 5-FU metabolite fluorodeoxyuridine monophosphate (FdUMP) forms a stable complex with TS and CH2THF resulting in enzyme inhibition (Longley et al., 2003). Recently, more specific folate-based inhibitors of TS have been developed such as tomudex (TDX) and Alimta (MTA), which form a stable complex with TS and dUMP that inhibits binding of CH_2THF to the enzyme (Hughes et al., 1999; Shih et al., 1997). TS inhibition causes nucleotide pool imbalances that result in S phase cell cycle arrest and apoptosis (Aherne et al., 1996; Longley et al., 2002; Longley et al., 2001). Oxaliplatin is a third generation platinum-based DNA damaging agent that is used in combination with 5-FU in the treatment of advanced colorectal cancer (Giacchetti et al., 2000).

Drug resistance is a major factor limiting the effectiveness of chemotherapies. Fas is a member of the tumour necrosis factor (TNF) receptor family. Binding of Fas Ligand (FasL) causes trimerization of Fas and leads to recruitment of the adaptor protein FADD (Fas-associated death domain), which in turn recruits procaspase 8 zymogens to from the death-inducing signalling complex (DISC) (Nagata, 1999). Procaspase 8 molecules become activated at the DISC and subsequently activate pro-apoptotic downstream molecules such as caspase 3 and BID. FasL expression is up-regulated in most colon tumours, and it has been postulated that tumour FasL induces apoptosis

of Fas-sensitive immune effector cells (O'Connell et al., 1999). This mechanism of immune escape requires that tumour cells develop resistance to Fas-mediated apoptosis to prevent autocrine and paracrine tumour cell death.

A key inhibitor of Fas signaling is c-FLIP, which inhibits procaspase 8 recruitment and processing at the DISC (Krueger et al., 2001). Differential splicing gives rise to long (c-FLIP$_L$) and short (c-FLIP$_S$) forms of c-FLIP, both of which bind to FADD within the DISC. c-FLIP$_S$ directly inhibits caspase 8 activation at the DISC, whereas c-FLIP$_L$ is first cleaved to a p43 truncated form that inhibits complete processing of procaspase 8 to its active subunits. c-FLIP also inhibits procaspase 8 activation at DISCs formed by the TRAIL (TNF-related apoptosis-inducing ligand) death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) (Krueger et al., 2001). In addition to blocking caspase 8 activation, DISC-bound c-FLIP has been reported to promote activation of the ERK, PI3-kinase/Akt and NF-κB signaling pathways (Krueger et al., 2001). Thus, c-FLIP potentially converts death receptor signaling from pro- to anti-apoptotic by activating intrinsic survival pathways. Significantly, c-FLIP$_L$ has been found to be overexpressed in colonic adenocarcinomas compared to matched normal tissue, suggesting that c-FLIP may contribute to in vivo tumour transformation (Ryu et al., 2001).

Summary of the Invention

As described herein and, as shown in our co-pending GB patent application entitled "treatment Medicament" and filed on the same day as the present application, the present inventors have surprisingly shown that by combining treatment using a death receptor ligand, such as an anti FAS antibody, for example, CH-11, with a chemotherapeutic agent such as 5-FU or an antifolate drug, such as ralitrexed (RTX) or pemetrexed (MTA, Alimta), a synergistic effect is achieved in the killing of cancer cells. However, the synergistic effect achieved was abrogated in cancer cells which overexpress c-FLIP.

The demonstration that high levels of c-FLIP expression in cancer cells inhibits drug induced apoptosis of such cells enables the determination prior to treatment of whether or not treatment with a particular drug regime may be effective in a particular patient. Thus, the present invention may be used in assays to determine whether or not treatment with a particular chemotherapeutic agent may be effective in a particular patient.

Accordingly, in a first aspect of the present invention, there is provided a method to predict response of tumour cells to in vivo treatment with a chemotherapeutic regime, said method comprising the steps:

(a) providing an in vitro sample containing tumour cells from a subject;

(b) determining the basal expression of one or more of the genes encoding c-FLIP protein, wherein enhanced expression of said gene correlates with enhanced resistance to the chemotherapeutic regime.

Basal expression in the tumour cells may be compared with basal expression in control samples. The control samples may be 5-FU sensitive, oxaliplatin sensitive and/or tomudex sensitive cancer cell-lines. For example, the control sample may be the H630 5-FU sensitive cancer cell line.

Alternatively, the control samples may be samples of cells from non-cancerous tissues of human subjects, preferably cancer-free human subjects. The basal expression level of the gene(s) in the control sample(s) may be determined in advance to provide control basal expression level value(s) with which to compare the expression level(s) of the in vitro sample.

As well as showing that overexpression of basal c-FLIP is associated with enhanced resistance to chemotherapeutic regimes, for example, with enhanced resistance to combined therapy comprising treatment with anti-Fas ligand, for example, CH-11, combined with a chemotherapeutic agent such as 5-FU or an antifolate drug, the inventors have further shown that basal expression of c-FLIP is enhanced in certain tumour cells in response to treatment with a chemotherapeutic regime.

Thus, in a second aspect of the present invention, there is provided a method for evaluating in vitro the response of tumour cells from a subject to the presence of a chemotherapeutic regime to predict response of the tumour cells in vivo to treatment with the chemotherapeutic regime, which method comprises:

(a) providing an in vitro sample containing tumour cells from a subject;

(b) exposing a portion of said sample of tumour cells to said chemotherapeutic regime;

(c) measuring expression of c-FLIP in said tumour cells; wherein enhanced expression of c-FLIP in response to said chemotherapeutic regime is indicative of enhanced resistance to said chemotherapeutic regime.

The presence of enhanced expression can be determined, for example, with reference to expression in a control portion of said sample which has not been exposed to said chemotherapeutic regime or to expression of said gene in the same sample prior to application of the chemotherapeutic regime.

In preferred embodiments of the invention, expression of c-FLIP in the sample exposed to said chemotherapeutic agent is considered to be enhanced if the expression is at least 2-fold, preferably at least 3-fold, more preferably at least 4-fold, even more preferably at least 5-fold, yet more preferably at least 10-fold, most preferably at least 12-fold that of c-FLIP in the control portion of said sample

which has not been exposed to said chemotherapeutic regime.

The chemotherapeutic regime may be any chemotherapeutic treatment suitable for treatment of tumours. For example, the regime may include treatment with one or more suitable chemotherapeutic agents and/or one or more anti-tumour specific binding members.

In one preferred embodiment, the chemotherapeutic regime does not consist of treatment with 5-FU, tomudex and/or oxaliplatin.

In particularly preferred embodiments of the invention, the chemotherapeutic regime comprises treatment using a death receptor ligand, such as an anti FAS antibody, for example, CH-11, combined with a chemotherapeutic agent such as 5-FU or an antifolate drug, such as ralitrexed (RTX) or pemetrexed (MTA, Alimta). As described herein, such combinations are strongly synergistic.

Such a treatment regime forms an independent aspect of the present invention.

As described in the Examples, in cell lines which demonstrated overexpression of c-FLIP and associated resistance to chemotherapy e.g 5-FU induced apoptosis, inhibition of FLIP expression reversed the resistance to chemotherapy -induced apoptosis.

Accordingly, in a third aspect, the invention provides a method of sensitising cancer cells to chemotherapy, said method comprising the step of administration to said cells a c-FLIP inhibitor.

Any suitable c-FLIP inhibitor may be used in methods of the invention. The inhibitor may be peptide or non-peptide.

In one preferred embodiment, said c-FLIP inhibitor is an antisense molecule which modulates the expression of the gene encoding c-FLIP.

In a more preferred embodiment, said c-FLIP inhibitor is an RNAi agent, which modulates expression of the c-FLIP gene. The agent may be an siRNA, an shRNA, a ddRNAi construct or a transcription template thereof, e.g., a DNA encoding an shRNA. In preferred embodiments the RNAi agent is an siRNA which is homologous to a part of the mRNA sequence of the gene encoding c-FLIP.

Indeed such an RNAi agent represents a fourth independent aspect of the present invention.

Preferred RNAi agents of and for use in the invention are between 15 and 25 nucleotides in length, preferably between 19 and 22 nucleotides, most preferably 21 nucleotides in length. In particularly preferred embodiments of the invention, the RNAi agent has the nucleotide seqence shown as SEQ ID NO: 1.

AAG CAG TCT GTT CAA GGA GCA (SEQ ID NO: 1)

According to a fifth aspect of the invention, there is provided a vector comprising an RNAi agent of the invention.

Furthermore, the invention may also be used to identify novel c-FLIP inhibitors, which may be used in the invention and which may be useful in chemotherapeutic treatments and regimes. Such agents may reduce or inhibit, either directly or indirectly, the effects of c-FLIP.

Accordingly, in a sixth aspect of the invention, there is provided an assay method for identifying a chemotherapeutic agent for use in the treatment of cancer, said method comprising the steps:

(a) providing a sample of tumour cells;

(b) exposing a portion of said sample to a candidate chemotherapeutic agent;

(c) determining expression of c-FLIP in said sample wherein a reduction in expression of c-FLIP compared to expression in a control sample is indicative of chemotherapeutic activity.

Expression in a control sample may be determined with reference to a different sample of said tumour cells which has not been exposed to said candidate agent or with reference to expression in the same sample prior to application of the candidate chemotherapeutic agent.

C-FLIP inhibitors of and for use in the invention may be used in *in vitro* and *in vivo* to kill cancer cells.

Thus, in a seventh aspect, the present invention provides a method of killing cancer cells comprising administration of a therapeutically effective amount of a c-FLIP inhibitor.

In an eighth aspect, the present invention provides a method of treating cancer comprising administration of a therapeutically effective amount of a c-FLIP inhibitor.

As described above, a C-FLIP inhibitor may be used to reverse or reduce resistance to chemotherapy-induced apoptosis.

In a tenth aspect, there is provided the use of a c-FLIP inhibitor in the preparation of a medicament for treating cancer.

According to an eleventh aspect, there is provided a pharmaceutical composition for the treatment of cancer, wherein the composition comprises a c-FLIP inhibitor and a pharmaceutically acceptable excipient, diluent or carrier.

The c-FLIP inhibitor may be administered alone or in combination with one or more further

chemotherapeutic substances. Such substances may be chemotherapeutic agents as described above or may be specific binding members with chemotherapeutic activity.

In particularly preferred embodiments of the invention, the c-FLIP inhibitor is administered as part of a treatment regime comprising
(a) a c-FLIP inhibitor and
(b) (i) a specific binding member which binds to a cell death receptor, or a nucleic acid encoding said binding member; and
(ii) a chemotherapeutic agent.

Thus, in a preferred aspect of the tenth aspect of the invention, there is provided the use of
(a) a c-FLIP inhibitor and
(b) (i) a specific binding member which binds to a cell death receptor, or a nucleic acid encoding said binding member; and/or
(ii) a chemotherapeutic agent in the preparation of a medicament for treating cancer.

Further in a preferred aspect of the eleventh aspect of the invention, there is provided a pharmaceutical composition for the treatment of cancer, wherein the composition comprises a) a c-FLIP inhibitor and
(b) (i) a specific binding member which binds to a cell death receptor, or a nucleic acid encoding said binding member; and/or
(ii) a chemotherapeutic agent and
(c) a pharmaceutically acceptable excipient, diluent

or carrier.

In an twelfth aspect, there is provided a product comprising:
a) a c-FLIP inhibitor and
(b) (i) a specific binding member which binds to a cell death receptor, or a nucleic acid encoding said binding member; and/or
(ii) a chemotherapeutic agent
as a combined preparation for the simultaneous, separate or sequential use in the treatment of cancer.

In a thirteenth aspect, there is provided a kit for the treatment of cancer, said kit comprising a) a c-FLIP inhibitor and
(b) (i) a specific binding member which binds to a cell death receptor, or a nucleic acid encoding said binding member; and/or
(ii) a chemotherapeutic agent and
(c) instructions for the administration of (a) and (b) separately, sequentially or simultaneously.

The c-FLIP inhibitor, the specific binding member and/or the chemotherapeutic agent may be administered simultaneously, sequentially or simultaneously. In preferred embodiments of the invention, the C-FLIP inhibitor is administered prior to the specific binding member and the chemotherapeutic agent.

A preferred binding member for use in the invention is an antibody or a fragment thereof. In particularly preferred embodiments, the binding member is the FAS antibody CH11 (Yonehara, S., Ishii, A. and Yonehara, M. (1989) J. Exp. Med. 169, 1747-1756) (available commercially e.g. from Upstate Biotechnology, Lake Placid, NY).

Any suitable chemotherapeutic agent may be used in the present invention. In preferred embodiments, the agent is doxorubicin, oxaliplatin, taxol, 5-Fluorouracil (5-FU), Irinotecan (CPT11) or an antifolate e.g. MTA or RTX. In one preferred embodiment, the agent is, 5-Fluorouracil, an antifolate (for example RTX or MTA), or a combination thereof. In a particularly preferred embodiment, the agent is 5-FU or an antifolate. Preferably, the agent is an antifolate. In a particularly preferred embodiment the agent is MTA.

In preferred embodiments of the invention, the c-FLIP inhibitor is used in combination with a specific binding member which binds to a cell death receptor as described above, or a nucleic acid encoding said binding member; and a chemotherapeutic agent.

In those embodiments in whih both are used, the concentrations of binding members and chemotherapeutic agents used are preferably sufficient to provide a synergistic effect.

Synergism is preferably defined as an RI of greater than unity using the method of Kern as modified by Romaneli (13, 14). The RI may be calculated as the ratio of expected cell survival (S_{exp}, defined as the product of the survival observed with drug A alone and the survival observed with drug B alone) to the observed cell survival (S_{obs}) for the combination of A and B ($RI=S_{exp}/S_{obs}$). Synergism may then be defined as an RI of greater than unity.

In preferred embodiments of the invention, said specific binding member and chemotherapeutic agent are provided in concentrations sufficient to produce an RI of greater than 1.5, more preferably greater than 2.0, most preferably greater than 2.25.

The combined medicament thus preferably produces a synergistic effect when used to treat tumour cells.

Preferred features of each aspect of the invention are as for each of the other aspects mutatis mutandis unless the context demands otherwise.

Detailed Description

As described above, the present invention relates to methods of screening samples comprising tumour cells for expression of particular genes in order to determine suitability for treatment using chemotherapeutic agents and methods of treatment of cancer.

The methods of the invention may involve the determination of expression of FLIP protein.

The expression of FLIP may be measured using any technique known in the art. Either mRNA or protein can be measured as a means of determining up-or down regulation of expression of a gene. Quantitative techniques are preferred. However semi-quantitative or qualitative techniques can also be used. Suitable techniques for measuring gene products include, but are not limited to, SAGE analysis, DNA microarray analysis, Northern blot, Western blot, immunocytochemical analysis, and ELISA.

In the methods of the invention, RNA can be detected using any of the known techniques in the art. Preferably an amplification step is used as the amount of RNA from the sample may be very small. Suitable techniques may include real-time RT-PCR, hybridisation of copy mRNA (cRNA) to an array of nucleic acid probes and Northern Blotting.

For example, when using mRNA detection, the method may be carried out by converting the isolated mRNA to cDNA according to standard methods; treating the converted cDNA with amplification reaction reagents (such as cDNA PCR reaction reagents) in a container along with an appropriate mixture of nucleic acid primers; reacting the contents of the container to produce amplification products; and analyzing the amplification products to detect the presence of

gene expression products of one or more of the genes encoding FLIP protein. Analysis may be accomplished using Southern Blot analysis to detect the presence of the gene products in the amplification product. Southern Blot analysis is known in the art. The analysis step may be further accomplished by quantitatively detecting the presence of such gene products in the amplification products, and comparing the quantity of product detected against a panel of expected values for known presence or absence in normal and malignant tissue derived using similar primers.

In e.g. determining gene expression in carrying out methods of the invention, conventional molecular biological, microbiological and recombinant DNA techniques techniques known in the art may be employed. Details of such techniques are described in, for example, Sambrook, Fritsch and Maniatis, "Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989, and Ausubel et al, Short Protocols in Molecular Biology, John Wiley and Sons, 1992).

The methods of the invention may be used to determine the suitability for treatment of any suitable cancer with a chemotherapeutic regime. For example the methods of the invention may be used to determine the sensitivity or resistance to treatment of cancers including, but not limited to, gastrointestinal, breast, prostate, head and neck cancers.

The nature of the tumour or cancer will determine the nature of the sample which is to be used in the methods of the invention. The sample may be, for example, a sample from a tumour tissue biopsy, bone marrow biopsy or circulating tumour cells in e.g. blood. Alternatively, e.g. where the tumour is a gastrointestinal tumour, tumour cells may be isolated from faeces samples. Other sources of tumour cells may include plasma, serum, cerebrospinal fluid, urine, interstitial fluid, ascites fluid etc.

For example, solid tumours may be collected in complete tissue culture medium with antibiotics. Cells may be manually teased from the tumour specimen or, where necessary, are enzymatically disaggregated by incubation with collagenase/DNAse and suspended in appropriate media containing, for example, human or animal sera.

In other embodiments, biopsy samples may be isolated and frozen or fixed in fixatives such as formalin. The samples may then be tested for expression levels of genes at a later stage.

Binding members

In the context of the present invention, a "binding member" is a molecule which has binding specificity for another molecule, in particular a receptor, preferably a death receptor. The binding member may

be a member of a pair of specific binding members. The members of a binding pair may be naturally derived or wholly or partially synthetically produced. One member of the pair of molecules may have an area on its surface, which may be a protrusion or a cavity, which specifically binds to and is therefore complementary to a particular spatial and polar organisation of the other member of the pair of molecules. Thus, the members of the pair have the property of binding specifically to each other. A binding member of the invention and for use in the invention may be any moiety, for example an antibody or ligand, which preferably can bind to a death receptor.

The binding member may bind to any death receptor. Death receptors include, Fas, TNFR, DR-3, DR-4 and DR-5. In preferred embodiments of the invention, the death receptor is FAS.

In preferred embodiments, the binding member comprises at least one human constant region.

Antibodies

An "antibody" is an immunoglobulin, whether natural or partly or wholly synthetically produced. The term also covers any polypeptide, protein or peptide having a binding domain which is, or is homologous to, an antibody binding domain. These can be derived from natural sources, or they may be partly or wholly synthetically produced. Examples of

antibodies are the immunoglobulin isotypes and their isotypic subclasses and fragments which comprise an antigen binding domain such as Fab, scFv, Fv, dAb, Fd; and diabodies.

A binding member for use in certain embodiments, the invention may be an antibody such as a monoclonal or polyclonal antibody, or a fragment thereof. The constant region of the antibody may be of any class including, but not limited to, human classes IgG, IgA, IgM, IgD and IgE. The antibody may belong to any sub class e.g. IgG1, IgG2, IgG3 and IgG4. IgG1 is preferred.

As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any binding member or substance having a binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or wholly or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023.

Examples of such fragments which can be used in the invention include the Fab fragment, the Fd fragment, the Fv fragment, the dAb fragment (Ward, E.S. et

al., Nature 341:544-546 (1989)), F(ab')2 fragments, single chain Fv molecules (scFv), bispecific single chain Fv dimers (PCT/US92/09965) and "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Hollinger et al., Proc. Natl. Acad. Sci. USA 90:6444-6448 (1993)).

A fragment of an antibody or of a polypeptide for use in the present invention generally means a stretch of amino acid residues of at least 5 to 7 contiguous amino acids, often at least about 7 to 9 contiguous amino acids, typically at least about 9 to 13 contiguous amino acids, more preferably at least about 20 to 30 or more contiguous amino acids and most preferably at least about 30 to 40 or more consecutive amino acids.

A "derivative" of such an antibody or polypeptide, or of a fragment antibody means an antibody or polypeptide modified by varying the amino acid sequence of the protein, e.g. by manipulation of the nucleic acid encoding the protein or by altering the protein itself. Such derivatives of the natural amino acid sequence may involve insertion, addition, deletion and/or substitution of one or more amino acids, preferably while providing a peptide having death receptor, e.g. FAS neutralisation and/or binding activity. Preferably such derivatives involve the insertion, addition, deletion and/or substitution of 25 or fewer amino acids, more preferably of 15 or fewer, even more preferably of

10 or fewer, more preferably still of 4 or fewer and most preferably of 1 or 2 amino acids only.

In preferred embodiments, the binding member is humanised. Methods for making humanised antibodies are known in the art e.g see U.S. Patent No. 5,225,539. A humanised antibody may be a modified antibody having the hypervariable region of a monoclonal antibody and the constant region of a human antibody. Thus the binding member may comprise a human constant region. The variable region other than the hypervariable region may also be derived from the variable region of a human antibody and/or may also be derived from a monoclonal antibody. In such case, the entire variable region may be derived from murine monoclonal antibody and the antibody is said to be chimerised. Methods for making chimerised antibodies are known in the art (e.g see U.S. Patent Nos. 4,816,397 and 4,816,567).

It is possible to take monoclonal and other antibodies and use techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementary determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP-A-184187, GB 2188638A or EP-A-239400. A hybridoma or

other cell producing an antibody may be subject to genetic mutation or other changes, which may or may not alter the binding specificity of antibodies produced.

A typical antibody for use in the present invention is a humanised equivalent of CH11 or any chimerised equivalent of an antibody that can bind to the FAS receptor and any alternative antibodies directed at the FAS receptor that have been chimerised and can be use in the treatment of humans. Furthermore, the typical antibody is any antibody that can cross-react with the extracellular portion of the FAS receptor and either bind with high affinity to the FAS receptor, be internalised with the FAS receptor or trigger signalling through the FAS receptor.

Production of Binding Members

Binding members, which my be used in the present invention may be generated wholly or partly by chemical synthesis. The binding members can be readily prepared according to well-established, standard liquid or, preferably, solid-phase peptide synthesis methods, general descriptions of which are broadly available (see, for example, in J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, 2nd edition, Pierce Chemical Company, Rockford, Illinois (1984), in M. Bodanzsky and A. Bodanzsky, The Practice of Peptide Synthesis, Springer Verlag, New York (1984); and Applied Biosystems 430A Users Manual, ABI Inc., Foster City, California), or they

may be prepared in solution, by the liquid phase method or by any combination of solid-phase, liquid phase and solution chemistry, e.g. by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the respective carbonic or sulfonic acid or a reactive derivative thereof.

Another convenient way of producing a binding member suitable for use in the present invention is to express nucleic acid encoding it, by use of nucleic acid in an expression system. Thus the present invention further provides the use of (a) nucleic acid encoding a specific binding member which binds to a cell death receptor and (b) a chemotherapeutic agent in the preparation of a medicament for treating cancer.

Nucleic acids of and/or for use in accordance with the present invention may comprise DNA or RNA and may be wholly or partially synthetic. In a preferred aspect, nucleic acid for use in the invention codes for a binding member of the invention as defined above. The skilled person will be able to determine substitutions, deletions and/or additions to such nucleic acids which will still provide a binding member suitable for use in the present invention.

Nucleic acid sequences encoding a binding member for use with the present invention can be readily prepared by the skilled person using the information

and references contained herein and techniques known in the art (for example, see Sambrook, Fritsch and Maniatis, "Molecular Cloning", A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989, and Ausubel et al, Short Protocols in Molecular Biology, John Wiley and Sons, 1992), given the nucleic acid sequences and clones available. These techniques include (i) the use of the polymerase chain reaction (PCR) to amplify samples of such nucleic acid, e.g. from genomic sources, (ii) chemical synthesis, or (iii) preparing cDNA sequences. DNA encoding antibody fragments may be generated and used in any suitable way known to those of skill in the art, including by taking encoding DNA, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. The portion may then be operably linked to a suitable promoter in a standard commercially available expression system. Another recombinant approach is to amplify the relevant portion of the DNA with suitable PCR primers. Modifications to the sequences can be made, e.g. using site directed mutagenesis, to lead to the expression of modified peptide or to take account of codon preferences in the host cells used to express the nucleic acid.

The nucleic acid may be comprised as construct(s) in the form of a plasmid, vector, transcription or expression cassette which comprises at least one nucleic acid as described above. The construct may be comprised within a recombinant host cell which

comprises one or more constructs as above. Expression may conveniently be achieved by culturing under appropriate conditions recombinant host cells containing the nucleic acid. Following production by expression a specific binding member may be isolated and/or purified using any suitable technique, then used as appropriate.

Binding members-encoding nucleic acid molecules and vectors for use in accordance with the present invention may be provided isolated and/or purified, e.g. from their natural environment, in substantially pure or homogeneous form, or, in the case of nucleic acid, free or substantially free of nucleic acid or genes of origin other than the sequence encoding a polypeptide with the required function.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, mammalian cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, NSO mouse melanoma cells and many others. A common, preferred bacterial host is E. coli.

The expression of antibodies and antibody fragments in prokaryotic cells such as E. coli is well established in the art. For a review, see for example Plückthun, Bio/Technology 9:545-551 (1991).

Expression in eukaryotic cells in culture is also available to those skilled in the art as an option for production of a binding member, see for recent review, for example Reff, Curr. Opinion Biotech. 4:573-576 (1993); Trill et al., Curr. Opinion Biotech. 6:553-560 (1995).

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual: 2nd Edition, Cold Spring Harbor Laboratory Press (1989). Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Ausubel et al. eds., Short Protocols in Molecular Biology, 2nd Edition, John Wiley & Sons (1992).

The nucleic acid may be introduced into a host cell by any suitable means. The introduction may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or,

for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells under conditions for expression of the gene.

The nucleic acid may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome in accordance with standard techniques. The nucleic acid may be on an extra-chromosomal vector within the cell, or otherwise identifiably heterologous or foreign to the cell.

RNAi agents

As described herein, c-FLIP inhibitors for use in the invention may be RNAi agents.

RNA interference (RNAi) or posttranscriptional gene silencing (PTGS) is a process whereby double-stranded RNA induces potent and specific gene

silencing. RNAi is mediated by RNA-induced silencing complex (RISC), a sequence-specific, multicomponent nuclease that destroys messenger RNAs homologous to the silencing trigger. RISC is known to contain short RNAs (approximately 22 nucleotides) derived from the double-stranded RNA trigger.

In one aspect, the invention provides methods of employing an RNAi agent to modulate expression, preferably reducing expression of a target gene, c-FLIP, in a mammalian, preferably human host. By reducing expression is meant that the level of expression of a target gene or coding sequence is reduced or inhibited by at least about 2-fold, usually by at least about 5-fold, e.g., 10-fold, 15-fold, 20-fold, 50-fold, 100-fold or more, as compared to a control. In certain embodiments, the expression of the target gene is reduced to such an extent that expression of the c-FLIP gene /coding sequence is effectively inhibited. By modulating expression of a target gene is meant altering, e.g., reducing, translation of a coding sequence, e.g., genomic DNA, mRNA etc., into a polypeptide, e.g., protein, product.

The RNAi agents that may be employed in preferred embodiments of the invention are small ribonucleic acid molecules (also referred to herein as interfering ribonucleic acids), that are present in duplex structures, e.g., two distinct oligoribonucleotides hybridized to each other or a single ribooligonucleotide that assumes a small

hairpin formation to produce a duplex structure. Preferred oligoribonucleotides are ribonucleic acids of not greater than 100 nt in length, typically not greater than 75 nt in length. Where the RNA agent is an siRNA, the length of the duplex structure typically ranges from about 15 to 30 bp, usually from about 20 and 29 bps, most preferably 21 bp. Where the RNA agent is a duplex structure of a single ribonucleic acid that is present in a hairpin formation, i.e., a shRNA, the length of the hybridized portion of the hairpin is typically the same as that provided above for the siRNA type of agent or longer by 4-8 nucleotides.

In certain embodiments, instead of the RNAi agent being an interfering ribonucleic acid, e.g., an siRNA or shRNA as described above, the RNAi agent may encode an interfering ribonucleic acid. In these embodiments, the RNAi agent is typically a DNA that encodes the interfering ribonucleic acid. The DNA may be present in a vector.

The RNAi agent can be administered to the host using any suitable protocol known in the art. For example, the nucleic acids may be introduced into tissues or host cells by viral infection, microinjection, fusion of vesicles, particle bombardment, or hydrodynamic nucleic acid administration.

DNA directed RNA interference (ddRNAi) is an RNAi technique which may be used in the methods of the invention. ddRNAi is described in U.S. 6,573,099 and

GB 2353282. ddRNAi is a method to trigger RNAi which involves the introduction of a DNA construct into a cell to trigger the production of double stranded (dsRNA), which is then cleaved into small interfering RNA (siRNA) as part of the RNAi process. ddRNAi expression vectors generally employ RNA polymerase III promoters (e.g. U6 or H1) for the expression of siRNA target sequences transfected in mammallian cells. siRNA target sequences generated from a ddRNAi expression cassette system can be directly cloned into a vector that does not contain a U6 promoter. Alternatively short single stranded DNA oligos containing the hairpin siRNA target sequence can be annealed and cloned into a vector downsteam of the pol III promoter. The primary advantages of ddRNAi expression vectors is that they allow for long term interference effects and minimise the natural interferon response in cells..

Antisense nucleic acids

As described herein, c-FLIP inhibitors for use in the invention may be anti-sense molecules or nucleic acid constructs that express such anti-sense molecules as RNA. The antisense molecules may be natural or synthetic. Synthetic antisense molecules may have chemical modifications from native nucleic acids. The antisense sequence is complementary to the mRNA of the targeted c-FLIP gene, and inhibits expression of the targeted gene products. Antisense molecules inhibit gene expression through various mechanisms, e.g. by reducing the amount of mRNA

available for translation, through activation of RNAse H, or steric hindrance. One or a combination of antisense molecules may be administered, where a combination may comprise multiple different sequences.

Antisense molecules may be produced by expression of all or a part of the c-FLIP sequence in an appropriate vector, where the transcriptional initiation is oriented such that an antisense strand is produced as an RNA molecule. Alternatively, the antisense molecule may be a synthetic oligonucleotide. Antisense oligonucleotides will generally be at least about 7, usually at least about 12, more usually at least about 16 nucleotides in length, and usually not more than about 50, preferably not more than about 35 nucleotides in length.

A specific region or regions of the endogenous c-FLIP sense strand mRNA sequence is chosen to be complemented by the antisense sequence. Selection of a specific sequence for the oligonucleotide may use an empirical method, where several candidate sequences are assayed for inhibition of expression of the target gene in an in vitro or animal model. A combination of sequences may also be used, where several regions of the mRNA sequence are selected for antisense complementation.

Antisense oligonucleotides may be chemically synthesized by methods known in the art (see Wagner

et al. (1993), supra, and Milligan et al., supra.) Preferred oligonucleotides are chemically modified from the native phosphodiester structure, in order to increase their intracellular stability and binding affinity. A number of such modifications have been described in the literature, which alter the chemistry of the backbone, sugars or heterocyclic bases. Among useful changes in the backbone chemistry are phosphorodiamidate linkages, methylphosphonates phosphorothioates; phosphorodithioates, where both of the non-bridging oxygens are substituted with sulfur; phosphoroamidites; alkyl phosphotriesters and boranophosphates. Achiral phosphate derivatives include 3'-O-5'-S-phosphorothioate, 3'-S-5'-O-phosphorothioate, 3'-CH2-5'-O-phosphonate and 3'-NH-5'-O-phosphoroamidate. Peptide nucleic acids may replace the entire ribose phosphodiester backbone with a peptide linkage. Sugar modifications may also be used to enhance stability and affinity.

Chemotherapeutic Agents

Any suitable chemotherapeutic agent or agents may be used in the present invention. For example, an agent for use in the invention may include but is not limited to: 5-Fluorouracil (5 FU), antifolates, for example RTX or MTA, Doxorubicin, taxol, Leucovorin, Irinotecan, Mitomycin C, Oxaliplatin, Raltitrexed, Tamoxifen or Cisplatin.

In particularly preferred embodiments, the agent is 5-FU or an antifolate. More preferably, the agent is an antifolate. In one preferred embodiment, the agent is MTA.

Treatment

Treatment" includes any regime that can benefit a human or non-human animal. The treatment may be in respect of an existing condition or may be prophylactic (preventative treatment). Treatment may include curative, alleviation or prophylactic effects.

"Treatment of cancer" includes treatment of conditions caused by cancerous growth and includes the treatment of neoplastic growths or tumours. Examples of tumours that can be treated using the invention are, for instance, sarcomas, including osteogenic and soft tissue sarcomas, carcinomas, e.g., breast-, lung-, bladder-, thyroid-, prostate-, colon-, rectum-, pancreas-, stomach-, liver-, uterine-, cervical and ovarian carcinoma, lymphomas, including Hodgkin and non-Hodgkin lymphomas, neuroblastoma, melanoma, myeloma, Wilms tumor, and leukemias, including acute lymphoblastic leukaemia and acute myeloblastic leukaemia, gliomas and retinoblastomas.

In preferred embodiments of the invention, the cancer is one or more of colorectal, breast ,

ovarian, cervical, gastric, lung, liver, skin and myeloid (e.g. bone marrow) cancer.

Administration

As described above, c-FLIP inhibitors of and for use in the present invention may be administered in any suitable way. Moreover they may be used in combination therapy with other treatments, for example, other chemotherapeutic agents or binding members. In such embodiments, the c-FLIP inhibitors or compositions of the invention may be administered simultaneously, separately or sequentially with another chemotherapeutic agent.

Where administered separately or sequentially, they may be administered within any suitable time period e.g. within 1, 2, 3, 6, 12, 24, 48 or 72 hours of each other. In preferred embodiments, they are administered within 6, preferably within 2, more preferably within 1, most preferably within 20 minutes of each other.

In a preferred embodiment, the c-FLIP inhibitors and/or compositions of the invention are administered as a pharmaceutical composition, which will generally comprise a suitable pharmaceutical excipient, diluent or carrier selected dependent on the intended route of administration.

The c-FLIP inhibitors and/or compositions of the invention may be administered to a patient in need of treatment via any suitable route.

Some suitable routes of administration include (but are not limited to) oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural) administration. Intravenous administration is preferred.

The C-FLIP inhibitor, product or composition may be administered in a localised manner to a tumour site or other desired site or may be delivered in a manner in which it targets tumour or other cells. Targeting therapies may be used to deliver the active agents more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons, for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

For intravenous, injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such

as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may comprise a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

The c-FLIP inhibitors and/or compositions of the invention may also be administered via microspheres, liposomes, other microparticulate delivery systems or sustained release formulations placed in certain tissues including blood. Suitable examples of sustained release carriers include semipermeable polymer matrices in the form of shared articles, e.g. suppositories or microcapsules. Implantable or microcapsular sustained release matrices include polylactides (US Patent No. 3, 773, 919; EP-A-0058481) copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al, Biopolymers 22(1): 547-556, 1985), poly (2-hydroxyethyl-methacrylate) or ethylene vinyl acetate (Langer et al, J. Biomed. Mater. Res. 15: 167-277, 1981, and Langer, Chem. Tech. 12:98-105, 1982). Liposomes containing the

polypeptides are prepared by well-known methods: DE 3,218, 121A; Epstein et al, PNAS USA, 82: 3688-3692, 1985; Hwang et al, PNAS USA, 77: 4030-4034, 1980; EP-A-0052522; E-A-0036676; EP-A-0088046; EP-A-0143949; EP-A-0142541; JP-A-83-11808; US Patent Nos 4,485,045 and 4,544,545. Ordinarily, the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal rate of the polypeptide leakage.

Examples of the techniques and protocols mentioned above and other techniques and protocols which may be used in accordance with the invention can be found in Remington's Pharmaceutical Sciences, 16th edition, Oslo, A. (ed), 1980.

Pharmaceutical Compositions

As described above, the present invention extends to a pharmaceutical composition for the treatment of cancer, the composition comprising a) a c-FLIP inhibitor b) a pharmaceutically acceptable excipient, diluent or carrier.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention may comprise, in addition to active ingredients, a pharmaceutically acceptable excipient, carrier, buffer stabiliser or other

materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. intravenous.

The formulation may be a liquid, for example, a physiologic salt solution containing non-phosphate buffer at pH 6.8-7.6, or a lyophilised powder.

Dose

The c-FLIP inhibitors or compositions of the invention are preferably administered to an individual in a "therapeutically effective amount", this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is ultimately within the responsibility and at the discretion of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners.

The invention will now be described further in the following non-limiting examples. Reference is made to the accompanying drawings in which:

Figure 1A illustrates Western blot analysis of Fas, FasL, procaspase 8, FADD, BID, Bcl-2, c-$FLIP_L$, c-$FLIP_S$, DcR3 and β-tubulin in MCF-7 cells 72 hours after treatment with 5μM 5-FU and 50nM TDX.

Figure 1B illustrates analysis of the interaction between Fas and FasL following treatment with 5μM 5-FU and 50nM TDX for 48 hours. Lysates were immunoprecipitated using a FasL polyclonal antibody and analysed by Western blot using a Fas monoclonal antibody.

Figure 1C illustrates analysis of the interaction between Fas and p43- c-$FLIP_L$ following treatment with 5μM 5-FU and 50nM TDX for 48 hours. Lysates were immunoprecipitated using the anti-Fas CH-11 monoclonal antibody and analysed by Western blot using a c-FLIP monoclonal antibody.

Figure 2A illustrates flow cytometry of MCF-7 cells treated with no drug (control), CH-11 alone (250ng/ml), 5-FU alone (5μM) for 96 hours, or co-treated with 5-FU for 72 hours followed by CH-11 for a further 24 hours.

Figure 2B illustrates flow cytometry of MCF-7 cells treated with no drug (control), CH-11 alone (250ng/ml), TDX alone (50nM) for 96 hours, or co-

treated with TDX for 72 hours followed by CH-11 for a further 24 hours.

Figure 2C illustrates Western blot analysis of Fas expression in MCF-7 cells treated with 5μM 5-FU for 48 hours. β-tubulin was assessed as a loading control.

Figure 2D illustrates flow cytometry of MCF-7 cells treated with no drug (control), CH-11 alone (250ng/ml), OXA alone (5μM) for 96 hours, or co-treated with OXA for 72 hours followed by CH-11 for a further 24 hours.

Figure 2E illustrates Western blot analysis of Fas, procaspase 8 and PARP expression in MCF-7 cells treated with 5μM 5-FU alone for 96 hours, or co-treated with 5-FU for 72 hours followed by CH-11 for a further 24 hours.

Figure 2F illustrates Western blot analysis examining the kinetics of caspase 8 activation and c-$FLIP_L$ processing in MCF-7 cells treated for 72 hours with 5μM 5-FU followed by 250ng/ml CH-11 for the indicated times.

Figure 3A illustrates Western blot analysis of Fas expression in HCT116 cells treated with 5-FU, TDX or OXA for 48 hours. Equal loading was assessed using a β-tubulin antibody.

Figure 3B illustrates Western blot analysis of procaspase 8 and PARP expression in HCT116 cells treated no drug (Con), 5μM 5-FU, 100nM TDX or 2μM OXA in the presence or absence of co-treatment with 200ng/ml CH-11. For each combined treatment the cells were pre-treated with chemotherapeutic drug for 24 hours followed by CH-11 for a further 24 hours.

Figure 4A illustrates Western blot of c-$FLIP_L$ expression in MCF-7 cells stably transfected with a FLIPL (FL) contruct or empty vector (EV).

Figure 4B illustrates MTT cell viability assays in EV68, FL44 and FL64 cells treated with 5μM 5-FU in combination with 250ng/ml CH-11. The combined treatment resulted in a synergistic decrease in cell viability in EV68 cells (RI=2.06), but not FL44 (RI=1.14) or FL64 (1.01) cells.

Figure 4C illustrates Western blot analysis of c-$FLIP_L$, procaspase 8 and PARP expression in EV68 and FL64 cells treated with no drug (Con) or 5μM 5-FU in the presence (+) or absence (-) of co-treatment with 250ng/ml CH-11. For each combined treatment, the cells were pre-treated with 5-FU for 72 hours followed by CH-11 for a further 24 hours.

Figure 5A illustrates MTT cell viability assays in EV68, FL44 and FL64 cells treated with 50nM TDX or 500nM MTA in the presence and absence of 250ng/ml CH-11. Combined TDX/CH-11 treatment resulted in a

synergistic decrease in cell viability in EV68 cells (RI=1.75), that was significantly reduced in FL44 (RI=1.22) or FL64 (RI=1.19) cells. Combined MTA/CH-11 treatment resulted in a synergistic decrease in cell viability in EV68 cells (RI=1.86), that was significantly reduced in FL44 (RI=1.29) and FL64 (RI=1.06) cells.

Figure 5B illustrates MTT cell viability assays in EV68, FL44 and FL64 cells treated with 2.5μM OXA in the presence and absence of 250ng/ml CH-11. Combined OXA/CH-11 treatment resulted in a synergistic decrease in cell viability in EV68 cells (RI=2.13), that was significantly reduced in FL64 (RI=1.22) or FL44 (1.19) cells.

Figure 5C Western blot analysis of procaspase 8 and PARP expression in EV68 and FL64 cells treated with 50nM TDX or 500nM MTA in the presence (+) or absence (-) of co-treatment with 250ng/ml CH-11.

Figure 5D illustrates Western blot analysis of procaspase 8 and PARP expression in EV68 and FL64 cells treated with 2.5μM OXA in the presence (+) or absence (-) of co-treatment with 250ng/ml CH-11. For each combined treatment, the cells were pre-treated with 5-FU for 72 hours followed by CH-11 for a further 24 hours.

Figure 6A illustrates c-FLIP$_L$ and c-FLIP$_S$ expression in HCT116 cells transfected with 0, 1 and 10nM FLIP-

targeted siRNA for 48 hours. Equal loading was assessed using a β-tubulin antibody.

Figure 6B illustrates MTT cell viability assays of HCT116 cells transfected with 5nM FLIP-targeted (FT) or scrambled control (SC) siRNA in the presence and absence of co-treatment with 5μM 5-FU. Combined treatment with 5-FU and FT siRNA resulted in a synergistic decrease in cell viability (RI=1.92, $p<0.0005$). No synergistic decrease in viability was observed in cells co-treated with 5-FU and SC siRNA (RI=0.98).

Figure 6C illustrates Western blot analysis of caspase 8 activation and PARP cleavage in HCT116 cells 48 hours after treatment with no drug, 5μM 5-FU or 100nM TDX in mock transfected cells (M), cells transfected with 1nM scrambled control (SC) and cells transfected with 1nM FLIP-targeted (FT) siRNA.

Figure 7A illustrates c-FLIP$_L$ and c-FLIP$_S$ expression in MCF-7 cells transfected with 10nM FLIP-targeted (FT) or scrambled control (SC) siRNA for 48 hours. Equal loading was assessed using a β-tubulin antibody.

Figure 7B illustrates MTT cell viability assays of MCF-7 cells transfected with 2.5nM FT siRNA in the presence and absence of co-treatment with 5μM 5-FU. The combined treatment resulted in a synergistic decrease in cell viability (RI=1.56, $p<0.005$).

Figure 7C Western blot analysis of PARP cleavage in MCF-7 cells 96 hours after treatment with 5-FU in the presence (+) and absence (-) of 10nM FLIP-targeted siRNA.

Figure 8 illustrates MTT cell viability assays of HCT116 cells transfected with 0.5nM FT or SC siRNA in the presence and absence of co-treatment with: Fig 8A 5μM 5-FU; Fig 8B 100nM TDX and Fig 8C 1μM OXA. Cells were assayed after 72 hours. Combined treatment with FT siRNA (but not SC siRNA) and each cytotoxic drug resulted in synergistic decreases in cell viability as indicated by the RI values ($p<0.0005$ for each combination).

Examples

MATERIALS AND METHODS

Cell Culture. All cells were maintained in 5% CO_2 at 37°C. MCF-7 cells were maintained in DMEM with 10% dialyzed bovine calf serum supplemented with 1mM sodium pyruvate, 2mM L-glutamine and 50μg/ml penicillin/streptomycin (from Life Technologies Inc., Paisley, Scotland). HCT116 cells were grown in McCoy's 5A medium (GIBCO) supplemented with 10% dialysed foetal calf serum, 50mg/ml penicillin-streptomycin, 2mM L-glutamine and 1mM sodium pyruvate. Stably transfected MCF-7 and HCT116 cell lines and 'mixed populations' of transfected cells were maintained in medium supplemented with 100μg/ml (MCF-7) or 1.5mg/ml (HCT116) G418 (from Life Technologies Inc).

Western Blotting. Western blots were performed as previously described (Longley et al., 2002). The Fas/CD95, Bcl-2 and BID (Santa Cruz Biotechnology, Santa Cruz, CA), caspase 8 (Oncogene Research Products, Darmstadt, Germany), PARP (Pharmingen, BD Biosciences, Oxford, England), c-FLIP (NF-6, Alexis, Bingham UK) DcR3 (Imgenex, San Diego, CA) mouse monoclonal antibodies were used in conjunction with a horseradish peroxidase (HRP)-conjugated sheep anti-mouse secondary antibody (Amersham, Little Chalfont, Buckinghamshire, England). FasL rabbit polyclonal antibody (Santa Cruz Biotechnology) was used in conjunction with an HRP-conjugated donkey anti-rabbit secondary antibody (Amersham). Equal loading was assessed using a β-tubulin mouse monoclonal primary antibody (Sigma).

Co-immunoprecipitation reactions. 250µl of Protein A (IgG) or Protein L (IgM) Sepharose beads (Sigma) and 1µg of the appropriate antibody were mixed at 4°C for 1 hour. Antibody-associated beads were washed three times with ELB buffer (250mM NaCl, 0.1% IPEGAL, 5mM EDTA, 0.5mM DTT, 50mM HEPES). Protein lysate (200-400µg) was then added, and the mixture rotated at 4°C for 1 hour. The beads were then washed in ELB buffer five times and resuspended in 100µl of Western sample buffer (250mM TRIS pH 6.8, 4% SDS, 2% glycerol, 0.02% bromophenol blue) containing 10% β-mercaptoethanol. The samples were then heated at 95°C for 5 minutes and centrifuged (5mins/4,000rpm/4°C). The supernatant was collected and analysed by Western blotting.

Cell Viability Assays. Cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay (Mosmann, 1983). To investigate drug-induced Fas-mediated apoptosis, cells were seeded at 2,000-5,000 cells per well on 96-well plates. After 24 hours, the cells were treated with a range of concentrations of 5-FU, TDX, MTA or OXA for 24-72 hours followed by the agonistic Fas monoclonal antibody, CH-11 (MBL, Watertown, MA) for a further 24-48 hours. To assess chemotherapy/siRNA interactions, 20,000-50,000 cells were seeded per well on 24-well plates. Twenty-four hours later, the cells were transfected with FLIP-targeted (FT) or scrambled siRNA (SC). Four hours after transfection, the cells were treated with a range of concentrations of each drug for a further 72-96 hours. MTT (0.5mg/ml) was added to each well and the cells were incubated at 37°C for a further 2 hours. The culture medium was removed and formazan crystals reabsorbed in 200μl (96-well) or 1ml (24-well) DMSO. Cell viability was determined by reading the absorbance of each well at 570nm using a microplate reader (Molecular Devices, Wokingham, England).

Flow Cytometric Analysis. Cells were seeded at $1x10^5$ per well of a 6-well tissue culture plate. After 24 hours, 5-FU, TDX or OXA was added to the medium and the cells cultured for a further 72 hours, after which time 250ng/ml CH-11 was added for 24 hours. DNA content of harvested cells was evaluated after

propidium iodide staining of cells using the EPICS XL Flow Cytometer (Coulter, Miami, Fl).

siRNA transfections. FLIP-targeted siRNA was designed using the Ambion siRNA target finder and design tool (www.ambion.com/techlib/misc/siRNA_finder.html) to inhibit both splice variants of c-FLIP. Both c-FLIP-targeted (FT) and scrambled control (SC) siRNA were obtained from Xeragon (Germantown, MD). The FT siRNA sequence used was: AAG CAG TCT GTT CAA GGA GCA. The SC siRNA sequence used was: AAT TCT CCG AAC GTG TCA CGT. siRNA transfections were performed on sub-confluent cells incubated in Optimem medium using the oligofectamine reagent (both from Life Technologies Inc) according to the manufacturer's instructions.

Statistical Analyses. The nature of the interaction between the chemotherapeutic drugs and CH-11 was determined by calculating the R index (RI), which was initially described by Kern and later modified by Romaneli (Kern et al., 1988; Romanelli et al., 1998). The RI is calculated as the ratio of expected cell survival (Sexp, defined as the product of the survival observed with drug A alone and the survival observed with drug B alone) to the observed cell survival (Sobs) for the combination of A and B (RI=Sexp/Sobs). Synergism is then defined as an RI of greater than unity. Romaneelli et al suggest that a synergistic interaction may be of pharmacological interest when RI values are around

2.0 (Romanelli et al., 1998). To further assess the statistical significance of the interactions, we designed a univariate ANOVA analysis using the SPSS software package. This was an additive model based on the null hypothesis that there was no interaction between the drugs.

RESULTS

c-FLIP$_L$ is up-regulated, processed and bound to Fas in response to 5-FU and TDX. Analysis of Fas expression in MCF-7 cells revealed that it was up-regulated by ~12-fold 72 hours after treatment with an IC60 dose 5-FU and was also highly up-regulated (by ~7-fold) in response to treatment with an IC60 dose (25nM) of TDX (Fig. 1A). FasL expression was unaffected by each drug treatment, but appeared to be highly expressed in these cells. Expression of FADD was also unaffected by drug treatment. Somewhat surprisingly, neither caspase 8, nor its substrate BID were activated in 5-FU- or TDX-treated cells as indicated by a lack of down-regulation of the levels of procaspase 8 or full-length BID (Fig. 1A). Bcl-2 was highly down-regulated in response to each agent. Interestingly, c-FLIP$_L$ but not c-FLIP$_S$ was up-regulated by drug treatment. Furthermore, c-FLIP$_L$ was processed to its p43-form indicative of its recruitment and processing at the DISC (Fig. 1A). Expression of the Fas decoy receptor DcR3 was unaltered by drug treatment in these cells.

To further investigate the apparent inhibition of capsase 8 activation in 5-FU- and TDX-treated cells, we analysed the interaction between Fas and FasL following drug treatment. Co-immunoprecipitation reactions demonstrated that there was increased Fas-FasL binding following drug treatment (Fig. 1B), suggesting that the inhibition of caspase 8 activation was occurring downstream of receptor ligation. In support of this, we found that drug treatment increased the interaction between Fas and p43- c-FLIP_L (Fig. 1C). These results suggested the involvement of c-FLIP_L in inhibiting drug-induced activation of Fas-mediated apoptosis in MCF-7 cells.

Activation of drug-induced apoptosis by the Fas-targeted antibody CH-11 coincides with processing of c-FLIP_L. Expression of FasL by activated T cells and NK cells induces apoptosis of Fas expressing target cells in vivo. To mimic the effects of these immune effector cells in vitro, the agonistic Fas monoclonal antibody CH-11 was used. Cells were treated with either 5-FU or TDX for 72 hours followed by 250ng/ml CH-11 treatment for 24 hours. We found that CH-11 alone had little effect on apoptosis (Figs. 2A and B). Treatment with 5-FU alone for 96 hours resulted in a modest ~2-fold induction of apoptosis in response to 5μM 5-FU (Fig. 2A). However, addition of CH-11 to 5-FU-treated cells resulted in a dramatic increase in apoptosis, with a ~55% of cells in the sub-G1/G0 apoptotic phase following co-treatment with 5μM 5-FU and CH-11. Similarly, the combination of TDX with CH-11

resulted in dramatic activation of apoptosis, with ~60% of cells in the sub-G1/G0 apoptotic phase following combined treatment with 25nM TDX and CH-11 (Fig. 2B). We also examined the effect of CH-11 on apoptosis induced by the DNA-damaging agent OXA, which also potently induces Fas expression in MCF-7 cells (Fig. 2C). Similar to its effect on 5-FU and TDX-treated cells, CH-11 induced apoptosis of OXA-treated cells, with ~50% of cells in the sub-G1/G0 apoptotic phase (Fig. 2D).

We subsequently analysed activation of the Fas pathway in MCF-7 cells following co-treatment with 5-FU and CH-11. As already noted, treatment with 5-FU alone resulted in dramatic up-regulation of Fas, but had no effect on caspase 8 activation (Fig. 2E). However, co-treatment of MCF-7 cells with 5-FU and CH-11 resulted in a dramatic activation of caspase 8 as indicated by complete loss of procaspase 8 (Fig. 2E). Furthermore, cleavage of PARP (poly(ADP) ribose polymerase), a hallmark of apoptosis, was only observed in MCF-7 cells co-treated with 5-FU and CH-11 (Fig. 2E). We next analysed the kinetics of caspase 8 activation in 5-FU and CH-11 co-treated cells. Caspase 8 was potently activated 12 hours after addition of CH-11 to 5-FU pre-treated cells (Fig. 2F). Importantly, this coincided with complete processing of c-FLIP$_L$ to its p43-form (Fig. 2F). By 24 hours after the addition of CH-11, neither procaspase 8 nor c-FLIP$_L$ (both its full-length and truncated forms) was detected.

We also examined the ability of CH-11 to activate apoptosis in the HCT116 colon cancer cell line. Fas was potently up-regulated in HCT116 cells 48 hours after treatment with 5-FU, TDX and OXA (Fig. 3A). Treatment with each drug alone or CH-11 alone for 48 hours failed to significantly activate caspase 8 or induce PARP cleavage (Fig. 3B). However, treatment with each drug for 24 hours followed by CH-11 for a further 24 hours resulted in activation of caspase 8 and PARP cleavage. Importantly, activation of caspase 8 correlated with processing of c-FLIP$_L$ in drug and CH-11 co-treated cells (Fig. 3B).

Overexpression of c-FLIP$_L$ inhibits chemotherapy-induced Fas-mediated cell death. To further investigate the role of c-FLIP$_L$ in regulating Fas-mediated apoptosis following drug treatment, we developed a panel of MCF-7 cell lines overexpressing c-FLIP$_L$. We developed cell lines with 5-10-fold increased c-FLIP$_L$ expression compared to cells transfected with empty vector (Fig. 4A). The c-FLIP$_L$ -overexpressing cell lines FL44 and FL64 and cells transfected with empty vector (EV68) were taken forward for further characterisation. Cell viability assays indicated that treatment of EV68 cells with 5-FU followed by CH-11 resulted in a highly synergistic decrease in cell viability (RI=2.06, $p<0.0005$) (Fig. 4B). However, no synergistic decrease in cell viability was observed in 5-FU and CH-11 co-treated FL44 or FL64 cells, with RI values of 1.14 and 1.01 respectively (Fig. 4B). Furthermore, 5-FU and CH-11 co-treatment resulted in

caspase 8 activation and PARP cleavage in EV68 cells (Fig. 4C). In contrast, c-FLIP$_L$ overexpression in FL64 cells abrogated both activation of caspase 8 and PARP cleavage in response to 5-FU and CH-11 co-treatment (Fig. 4C).

We next examined the effect of c-FLIP$_L$ overexpression on Fas-mediated apoptosis following treatment with the antifolates TDX and MTA and the DNA-damaging agent OXA. All three drugs synergistically decreased cell viability in EV68 cells when combined with CH-11 (Figs. 5A and B). However, this synergistic interaction was inhibited by c-FLIP$_L$ overexpression in both the FL44 and FL64 cell lines (Figs. 5A and B). Analysis of caspase 8 activation and PARP cleavage confirmed that Fas-mediated apoptosis in response to all three agents was attenuated by c-FLIP$_L$ overexpression. Combined treatment with each antifolate and CH-11 resulted in caspase 8 activation in EV68 cells, but not FL64 cells (Fig. 5C). Similarly, PARP cleavage in response to the antifolates and CH-11 was inhibited in the FL64 cell line (Fig. 5C). Although some caspase 8 activation and PARP cleavage were observed in FL64 cells following co-treatment with 5μM OXA and CH-11, this was much reduced compared to the EV68 cell line (Fig. 5D). These results indicate that c-FLIP$_L$ is a key regulator of Fas-mediated apoptosis in response to 5-FU, antifolates and oxaliplatin.

siRNA-targeting of c-FLIP sensitises cancer cells to chemotherapy. Having established that c-FLIP$_L$ overexpression protected MCF-7 and HCT116 cells from chemotherapy-induced Fas-mediated cell death, we next designed a FLIP-targeted (FT) siRNA to inhibit both c-FLIP splice variants. Transfection with 10nM FT siRNA potently down-regulated expression of both c-FLIP splice variants in MCF-7 cells (Fig. 6A). Cell viability analysis of MCF-7 cells transfected with FT siRNA indicated that co-treatment with 5-FU resulted in a supra-additive decrease in cell viability (Fig. 6B, RI=1.56, $p<0.005$). Interestingly, transfection of MCF-7 cells with FT siRNA significantly decreased cell viability in the absence of co-treatment with 5-FU, with an approximate 50% decrease in cell viability in cells transfected with 2.5nM FT siRNA (Fig. 6B). A scrambled control (SC) siRNA that had no effect of FLIP expression, also had no effect on cell viability either alone or in combination with 5-FU (data not shown). The decrease in cell viability in response to FT siRNA alone appeared to be due to the induction of apoptosis, as transfection of FT siRNA in the absence of co-treatment with drug induced significant levels of PARP cleavage (Fig. 6C, lane 2). Furthermore, combined treatment with FT siRNA and 5-FU resulted in potent cleavage of PARP (Fig. 6C), indicating that the synergistic decrease in cell viability observed in MCF-7 cells co-treated with these agents was due to increased apoptosis.

FT siRNA also potently down-regulated $FLIP_L$ and $FLIP_S$ expression in HCT116 cells (Fig. 7A). Analysis of caspase 8 activation in siRNA-transfected HCT116 cells indicated that FT siRNA alone (1nM) caused some activation of caspase 8, as indicated by the decrease in the levels of p53/55 zymogen and appearance of the p41/43 cleavage products (Fig. 7B, lane 3). This was accompanied by some PARP cleavage. At higher concentrations (>5nM), FT siRNA alone caused more potent activation of caspase 8 and PARP cleavage in HCT116 cells (Fig. 7C). Both 5-FU (5μM) and TDX (100nM) caused some caspase 8 activation in mock and SC transfected HCT116 cells as indicated by the presence of p41/p43 caspase 8, although no PARP cleavage was observed in these cells (Fig. 7B). The most potent activation of caspase 8 was observed in cells co-treated with 1nM FT siRNA and 5-FU or TDX, with decreased expression of the p53/55 zymogen and increased expression of both the p41/43 and p18 caspase 8 cleavage products (Fig. 7B, lanes 6 and 9). Furthermore, activation of caspase 8 in FT siRNA/chemotherapy-treated HCT116 cells was accompanied by potent PARP cleavage. Cell viability assays indicated that co-treatment with 0.5nM FT siRNA and 5μM 5-FU resulted in a synergistic decrease in cell viability (Fig. 8A, RI=2.10, p<0.0005). In contrast, SC siRNA had no significant effect on cell viability either in the presence or absence of 5-FU. Furthermore, co-treatment with FT siRNA and both TDX and OXA resulted in synergistic decreases in cell viability, with RI values of 1.68 and 2.26 respectively (Figs. 8B and C). These

results indicate that inhibition of c-FLIP expression in HCT116 and MCF-7 cells dramatically sensitised them to chemotherapy-induced apoptosis.

DISCUSSION

We found that the Fas death receptor was highly up-regulated in response to 5-FU, the TS-targeted antifolates TDX and MTA and the DNA-damaging agent OXA in MCF-7 breast cancer and HCT116 colon cancer cells, however, this did not result in significant activation of apoptosis. Expression of FasL by activated T cells and natural killer cells induces apoptosis of Fas expressing target cells in vivo (O'Connell et al., 1999). To mimic the effects of these immune effector cells in our in vitro model, we used the agonistic Fas monoclonal antibody CH-11. We found that CH-11 potently activated apoptosis of chemotherapy-treated cells, suggesting that the Fas signalling pathway is an important mediator of apoptosis in response to these agents in vivo. Many tumour cells overexpress FasL, and it has been postulated that tumour FasL induces apoptosis of Fas-sensitive immune effector cells, thereby inhibiting the antitumor immune response (O'Connell et al., 1999). This hypothesis has been supported by both in vitro and in vivo studies (Bennett et al., 1998; O'Connell et al., 1997). The strategy of overexpressing FasL requires that the tumour cells develop resistance to Fas-mediated apoptosis to prevent autocrine and paracrine induction of tumour

cell death. The lack of caspase 8 activation that we observed in response to chemotherapy suggests that Fas-mediated apoptosis may be inhibited in MCF-7 and HCT116 and cancer cells, but that co-treatment with CH-11 was sufficient to overcome this resistance and activate Fas-mediated apoptosis.

Fas signalling may be inhibited by c-FLIP, which can inhibit caspase 8 recruitment to and activation at the Fas DISC (Krueger et al., 2001). Multiple c-FLIP splice variants have been reported, however, only two forms (c-FLIP$_L$ and c-FLIP$_S$) have been detected at the protein level (Scaffidi et al., 1999). Both splice variants have death effector domains (DEDs), with which they bind to FADD, blocking access of procaspase 8 molecules to the DISC. c-FLIP$_L$ is processed at the DISC as it is a natural substrate for caspase 8, which cleaves it to generate a truncated form of approximately 43kDa (p43-FLIPL) (Niikura et al., 2002). Cleaved p43- c-FLIP$_L$ binds more tightly to the DISC than full-length c-FLIP$_L$. c-FLIP$_S$ is not processed by caspase 8 at the DISC. c-FLIP$_L$ appears to be a more potent inhibitor of Fas-mediated cell death than c-FLIP$_S$ (Irmler et al., 1997; Tschopp et al., 1998). Initially both pro-apoptotic and anti-apoptotic effects were proposed for c-FLIP. However, enhanced cell death occurred mainly in experiments using transient over-expression and may have been due to excessive levels of these DED-containing proteins, which may have caused clustering of other DED-containing proteins including procaspase 8, resulting in caspase

activation (Siegel et al., 1998). The data from cell lines stably over-expressing c-FLIP and from mice deficient in c-FLIP support an anti-apoptotic function for c-FLIP (Yeh et al., 2000).

We found that c-$FLIP_L$ was up-regulated and processed to its p43-form in MCF-7 cells following treatment with 5-FU and TDX. Furthermore, activation of caspase 8 and apoptosis in cells co-treated with chemotherapy and CH-11 coincided with processing of c-$FLIP_L$. These results suggested that c-$FLIP_L$ regulated the onset of drug-induced Fas-mediated apoptosis in these cell lines. This hypothesis was further supported by data from overexpression and siRNA studies. c-FLIP overexpression abrogated the synergistic interaction between CH-11 and 5-FU, TDX, MTA and OXA by inhibiting caspase 8 activation. Furthermore, siRNA-targeting of both c-FLIP splice variants sensitised cells to these chemotherapeutic agents as determined by cell viability and PARP cleavage assays. Collectively, these results indicate that c-FLIP inhibts apoptosis in response to these drugs.

Interestingly, we also found that siRNA-mediated down-regulation of c-$FLIP_L$ and c-$FLIP_S$ induced caspase 8 activation and PARP cleavage in the absence of co-treatment with chemotherapy (although co-treatment with drug enhanced the effect). The mechanism of FLIP-targeted siRNA-mediated activation of apoptosis is currently being investigated. In addition to blocking caspase 8 activation, DISC-

bound c-FLIP has been reported to promote activation of the ERK, PI3-kinase/Akt and NFκB signalling pathways (Kataoka et al., 2000; Panka et al., 2001). The NFκB, PI3K/Akt and ERK signal transduction pathways are associated with cell survival and/or proliferation, therefore, c-FLIP is capable of both blocking caspase 8 activation and also recruiting adaptor proteins that can activate intrinsic survival and proliferation pathways (Shu et al., 1997). Furthermore, c-FLIP also inhibits procaspase 8 activation at the DISCs formed by the TRAIL receptors DR4 and DR5 (Krueger et al., 2001). rTRAIL induces apoptosis in a range of human cancer cell lines including colorectal and breast, indicating that the TRAIL receptors are widely expressed in tumour cells (Ashkenazi, 2002). It is possible that expression of DR4 and DR5 is tolerated in tumours because c-FLIP converts the apoptotic signal to one which promotes survival and proliferation. Thus, siRNA-mediated down-regulation of c-FLIP may induce apoptosis by inhibiting FLIP-mediated activation of NFκB, PI3K/Akt and ERK and promoting activation of caspase 8 at TRAIL DISCs.

In conclusion, we have found that c-FLIP is a key regulator of Fas-mediated apoptosis in response to 5-FU, TS-targeted antifolates and OXA. Our results suggest that c-FLIP may be a clinically useful predictive marker of response to these agents and that c-FLIP is a therapeutically attractive target.

All documents referred to in this specification are

herein incorporated by reference. Various modifications and variations to the described embodiments of the inventions will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes of carrying out the invention which are obvious to those skilled in the art are intended to be covered by the present invention.

REFERENCES

Aherne, G. W., Hardcastle, A., Raynaud, F., and Jackman, A. L. (1996). Immunoreactive dUMP and TTP pools as an index of thymidylate synthase inhibition; effect of tomudex (ZD1694) and a nonpolyglutamated quinazoline antifolate (CB30900) in L1210 mouse leukaemia cells. Biochem Pharmacol 51, 1293-1301.

Ashkenazi, A. (2002). Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat Rev Cancer 2, 420-430.

Bennett, M. W., O'Connell, J., O'Sullivan, G. C., Brady, C., Roche, D., Collins, J. K., and Shanahan, F. (1998). The Fas counterattack in vivo: apoptotic depletion of tumor-infiltrating lymphocytes associated with Fas ligand expression by human esophageal carcinoma. J Immunol 160, 5669-5675.

Giacchetti, S., Perpoint, B., Zidani, R., Le Bail, N., Faggiuolo, R., Focan, C., Chollet, P., Llory, J. F., Letourneau, Y., Coudert, B., et al. (2000). Phase III multicenter randomized trial of oxaliplatin added to chronomodulated fluorouracil-leucovorin as first-line treatment of metastatic colorectal cancer. J Clin Oncol 18, 136-147.

Hughes, L. R., Stephens, T. C., Boyle, F. T., and Jackman, A. L. (1999). Raltitrexed (TomudexTM), a highly polyglutamatable antifolate thymidylate synthase inhibitor, Humana press).

Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J. L., Schroter, M., Burns, K., Mattmann, C., et al. (1997). Inhibition of death receptor signals by cellular FLIP. Nature 388, 190-195.

Kataoka, T., Budd, R. C., Holler, N., Thome, M., Martinon, F., Irmler, M., Burns, K., Hahne, M., Kennedy, N., Kovacsovics, M., and Tschopp, J. (2000). The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. Curr Biol 10, 640-648.

Kern, D. H., Morgan, C. R., and Hildebrand-Zanki, S. U. (1988). In vitro pharmacodynamics of 1-beta-D-arabinofuranosylcytosine: synergy of antitumor activity with cis-diamminedichloroplatinum(II). Cancer Res 48, 117-121.

Krueger, A., Baumann, S., Krammer, P. H., and Kirchhoff, S. (2001). FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. Mol Cell Biol 21, 8247-8254.

Longley, D. B., Boyer, J., Allen, W. L., Latif, T., Ferguson, P. R., Maxwell, P. J., McDermott, U., Lynch, M., Harkin, D. P., and Johnston, P. G. (2002). The role of thymidylate synthase induction in modulating p53-regulated gene expression in response to 5-fluorouracil and antifolates. Cancer Res 62, 2644-2649.

Longley, D. B., Ferguson, P. R., Boyer, J., Latif, T., Lynch, M., Maxwell, P., Harkin, D. P., and Johnston, P. G. (2001). Characterization of a thymidylate synthase (TS)-inducible cell line: a model system for studying sensitivity to TS- and non-TS-targeted chemotherapies. Clin Cancer Res 7, 3533-3539.

Longley, D. B., Harkin, D. P., and Johnston, P. G. (2003). 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 3, 330-338.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65, 55-63.

Nagata, S. (1999). Fas ligand-induced apoptosis. Annu Rev Genet 33, 29-55.

Niikura, Y., Nonaka, T., and Imajoh-Ohmi, S. (2002). Monitoring of caspase-8/FLICE processing and activation upon Fas stimulation with novel antibodies directed against a cleavage site for caspase-8 and its substrate, FLICE-like inhibitory protein (FLIP). J Biochem (Tokyo) 132, 53-62.

O'Connell, J., Bennett, M. W., O'Sullivan, G. C., Collins, J. K., and Shanahan, F. (1997). The Fas

counterattack: a molecular mechanism of tumor immune privilege. Mol Med 3, 294-300.

O'Connell, J., Bennett, M. W., O'Sullivan, G. C., Collins, J. K., and Shanahan, F. (1999). Resistance to Fas (APO-1/CD95)-mediated apoptosis and expression of Fas ligand in esophageal cancer: the Fas counterattack. Dis Esophagus 12, 83-89.

Panka, D. J., Mano, T., Suhara, T., Walsh, K., and Mier, J. W. (2001). Phosphatidylinositol 3-kinase/Akt activity regulates c-FLIP expression in tumor cells. J Biol Chem 276, 6893-6896.

Romanelli, S., Perego, P., Pratesi, G., Carenini, N., Tortoreto, M., and Zunino, F. (1998). In vitro and in vivo interaction between cisplatin and topotecan in ovarian carcinoma systems. Cancer Chemother Pharmacol 41, 385-390.

Ryu, B. K., Lee, M. G., Chi, S. G., Kim, Y. W., and Park, J. H. (2001). Increased expression of cFLIP(L) in colonic adenocarcinoma. J Pathol 194, 15-19.

Scaffidi, C., Schmitz, I., Krammer, P. H., and Peter, M. E. (1999). The role of c-FLIP in modulation of CD95-induced apoptosis. J Biol Chem 274, 1541-1548.

Shih, C., Chen, V. J., Gossett, L. S., Gates, S. B., MacKellar, W. C., Habeck, L. L., Shackelford, K. A., Mendelsohn, L. G., Soose, D. J., Patel, V. F., et al. (1997). LY231514, a pyrrolo[2,3-d]pyrimidine-based antifolate that inhibits multiple folate-requiring enzymes. Cancer Res 57, 1116-1123.

Shu, H. B., Halpin, D. R., and Goeddel, D. V. (1997). Casper is a FADD- and caspase-related inducer of apoptosis. Immunity 6, 751-763.

Siegel, R. M., Martin, D. A., Zheng, L., Ng, S. Y., Bertin, J., Cohen, J., and Lenardo, M. J. (1998). Death-effector filaments: novel cytoplasmic structures that recruit caspases and trigger apoptosis. J Cell Biol 141, 1243-1253.

Tschopp, J., Irmler, M., and Thome, M. (1998). Inhibition of fas death signals by FLIPs. Curr Opin Immunol 10, 552-558.

Yeh, W. C., Itie, A., Elia, A. J., Ng, M., Shu, H. B., Wakeham, A., Mirtsos, C., Suzuki, N., Bonnard, M., Goeddel, D. V., and Mak, T. W. (2000). Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. Immunity 12, 633-642.

Aherne, G. W., Hardcastle, A., Raynaud, F., and Jackman, A. L. (1996). Immunoreactive dUMP and TTP pools as an index of thymidylate synthase inhibition; effect of tomudex (ZD1694) and a nonpolyglutamated quinazoline antifolate (CB30900) in L1210 mouse leukaemia cells. Biochem Pharmacol 51, 1293-1301.

Bennett, M., Macdonald, K., Chan, S. W., Luzio, J. P., Simari, R., and Weissberg, P. (1998a). Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. Science 282, 290-293.

Bennett, M. W., O'Connell, J., O'Sullivan, G. C., Brady, C., Roche, D., Collins, J. K., and Shanahan, F. (1998b). The Fas counterattack in vivo: apoptotic depletion of tumor-infiltrating lymphocytes associated with Fas ligand expression by human esophageal carcinoma. J Immunol 160, 5669-5675.

Bunz, F., Hwang, P. M., Torrance, C., Waldman, T., Zhang, Y., Dillehay, L., Williams, J., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1999). Disruption of p53 in human cancer cells alters the responses to therapeutic agents. J Clin Invest 104, 263-269.

Chou, T. C., and Talalay, P. (1984). Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22, 27-55.

Chu, E., Koeller, D. M., Johnston, P. G., Zinn, S., and Allegra, C. J. (1993). Regulation of thymidylate synthase in human colon cancer cells treated with 5-fluorouracil and interferon-gamma. Mol Pharmacol 43, 527-533.

Giacchetti, S., Perpoint, B., Zidani, R., Le Bail, N., Faggiuolo, R., Focan, C., Chollet, P., Llory, J. F., Letourneau, Y., Coudert, B., et al. (2000). Phase III multicenter randomized trial of oxaliplatin added to chronomodulated fluorouracil-leucovorin as first-line treatment of metastatic colorectal cancer. J Clin Oncol 18, 136-147.

Hughes, L. R., Stephens, T. C., Boyle, F. T., and Jackman, A. L. (1999). Raltitrexed (TomudexTM), a highly polyglutamatable antifolate thymidylate synthase inhibitor, Humana press).

Ichikawa, K., Yoshida-Kato, H., Ohtsuki, M., Ohsumi, J., Yamaguchi, J., Takahashi, S., Tani, Y., Watanabe, M., Shiraishi, A., Nishioka, K., et al. (2000). A novel murine anti-human Fas mAb which mitigates lymphadenopathy without hepatotoxicity. Int Immunol 12, 555-562.

Jiang, S., Song, M. J., Shin, E. C., Lee, M. O., Kim, S. J., and Park, J. H. (1999). Apoptosis in human hepatoma cell lines by chemotherapeutic drugs via Fas-dependent and Fas-independent pathways. Hepatology 29, 101-110.

Kano, Y., Ohnuma, T., Okano, T., and Holland, J. F. (1988). Effects of vincristine in combination with methotrexate and other antitumor agents in human acute lymphoblastic leukemia cells in culture. Cancer Res 48, 351-356.

Kern, D. H., Morgan, C. R., and Hildebrand-Zanki, S. U. (1988). In vitro pharmacodynamics of 1-beta-D-arabinofuranosylcytosine: synergy of antitumor activity with cis-diamminedichloroplatinum(II). Cancer Res 48, 117-121.

Krueger, A., Baumann, S., Krammer, P. H., and Kirchhoff, S. (2001). FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. Mol Cell Biol 21, 8247-8254.

Longley, D. B., Boyer, J., Allen, W. L., Latif, T., Ferguson, P. R., Maxwell, P. J., McDermott, U., Lynch, M., Harkin, D. P., and Johnston, P. G. (2002a). The role of thymidylate synthase induction in modulating p53-regulated gene expression in response to 5-fluorouracil and antifolates. Cancer Res 62, 2644-2649.

Longley, D. B., Ferguson, P. R., Boyer, J., Latif, T., Lynch, M., Maxwell, P., Harkin, D. P., and Johnston, P. G. (2001). Characterization of a thymidylate synthase (TS)-inducible cell line: a model system for studying sensitivity to TS- and

non-TS-targeted chemotherapies. Clin Cancer Res 7, 3533-3539.

Longley, D. B., Harkin, D. P., and Johnston, P. G. (2003). 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 3, 330-338.

Longley, D. B., McDermott, U., and Johnston, P. G. (2002b). Clinical significance of prognostic and predictive markers in colorectal cancer. Pharmacogenomics J 2, 209-216.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65, 55-63.

Muller, M., Wilder, S., Bannasch, D., Israeli, D., Lehlbach, K., Li-Weber, M., Friedman, S. L., Galle, P. R., Stremmel, W., Oren, M., and Krammer, P. H. (1998). p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. J Exp Med 188, 2033-2045.

Munsch, D., Watanabe-Fukunaga, R., Bourdon, J. C., Nagata, S., May, E., Yonish-Rouach, E., and Reisdorf, P. (2000). Human and mouse Fas (APO-1/CD95) death receptor genes each contain a p53-responsive element that is activated by p53 mutants unable to induce apoptosis. J Biol Chem 275, 3867-3872.

Nagata, S. (1999). Fas ligand-induced apoptosis. Annu Rev Genet 33, 29-55.

O'Connell, J., Bennett, M. W., O'Sullivan, G. C., Collins, J. K., and Shanahan, F. (1997). The Fas counterattack: a molecular mechanism of tumor immune privilege. Mol Med 3, 294-300.

O'Connell, J., Bennett, M. W., O'Sullivan, G. C., Collins, J. K., and Shanahan, F. (1999). Resistance to Fas (APO-1/CD95)-mediated apoptosis and expression of Fas ligand in esophageal cancer: the Fas counterattack. Dis Esophagus 12, 83-89.

Pitti, R. M., Marsters, S. A., Lawrence, D. A., Roy, M., Kischkel, F. C., Dowd, P., Huang, A., Donahue, C. J., Sherwood, S. W., Baldwin, D. T., et al. (1998). Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. Nature 396, 699-703.

Romanelli, S., Perego, P., Pratesi, G., Carenini, N., Tortoreto, M., and Zunino, F. (1998). In vitro and in vivo interaction between cisplatin and topotecan in ovarian carcinoma systems. Cancer Chemother Pharmacol 41, 385-390.

Ruberti, G., Cascino, I., Papoff, G., and Eramo, A. (1996). Fas splicing variants and their effect on apoptosis. Adv Exp Med Biol 406, 125-134.

Ryu, B. K., Lee, M. G., Chi, S. G., Kim, Y. W., and Park, J. H. (2001). Increased expression of cFLIP(L) in colonic adenocarcinoma. J Pathol 194, 15-19.

Sato, T., Irie, S., Kitada, S., and Reed, J. C. (1995). FAP-1: a protein tyrosine phosphatase that associates with Fas. Science 268, 411-415.

Shih, C., Chen, V. J., Gossett, L. S., Gates, S. B., MacKellar, W. C., Habeck, L. L., Shackelford, K. A., Mendelsohn, L. G., Soose, D. J., Patel, V. F., et al. (1997). LY231514, a pyrrolo[2,3-d]pyrimidine-based antifolate that inhibits multiple folate-requiring enzymes. Cancer Res 57, 1116-1123.

Swain, S. M., Lippman, M. E., Egan, E. F., Drake, J. C., Steinberg, S. M., and Allegra, C. J. (1989). Fluorouracil and high-dose leucovorin in previously treated patients with metastatic breast cancer. J Clin Oncol 7, 890-899.

Tillman, D. M., Petak, I., and Houghton, J. A. (1999). A Fas-dependent component in 5-fluorouracil/leucovorin-induced cytotoxicity in colon carcinoma cells. Clin Cancer Res 5, 425-430.

Claims

1. A method to predict response of tumour cells to in vivo treatment with a chemotherapeutic regime, said method comprising the steps:
(a) providing an in vitro sample containing tumour cells from a subject;
(b) determining the basal expression of one or more of the genes encoding c-FLIP protein, wherein enhanced expression of said gene correlates with enhanced resistance to the chemotherapeutic regime.

2. A method for evaluating in vitro the response of tumour cells from a subject to the presence of a chemotherapeutic regime to predict response of the tumour cells in vivo to treatment with the chemotherapeutic regime, which method comprises:
(a) providing an in vitro sample containing tumour cells from a subject;
(b) exposing a portion of said sample of tumour cells to said chemotherapeutic regime;
(c) measuring expression of c-FLIP in said tumour cells; wherein enhanced expression of c-FLIP in response to said chemotherapeutic regime is indicative of enhanced resistance to said chemotherapeutic regime.

3. The method according to claim 1 or claim 2, wherein the chemotherapeutic regime comprises treatment using a death receptor ligand

combined with a chemotherapeutic agent.

4. The method according to claim 3 wherein the death receptor ligand is CH-11 and the chemotherapeutic agent is 5-FU or an antifolate drug.

5. A method of sensitising cancer cells to chemotherapy, said method comprising the step of administration to said cells a c-FLIP inhibitor.

6. An assay method for identifying a chemotherapeutic agent for use in the treatment of cancer, said method comprising the steps:

(a) providing a sample of tumour cells;

(b) exposing a portion of said sample to a candidate chemotherapeutic agent;

(c) determining expression of c-FLIP in said sample wherein a reduction in expression of c-FLIP compared to expression in a control sample is indicative of chemotherapeutic activity.

7. A method of killing cancer cells comprising administration of a therapeutically effective amount of a c-FLIP inhibitor.

8. A method of treating cancer comprising administration of a therapeutically effective

amount of a c-FLIP inhibitor.

9. The method according to any one of claims 5 to 8, wherein the c-FLIP inhibitor is administered as part of a treatment regime comprising
(a) a c-FLIP inhibitor and
(b) (i) a specific binding member which binds to a cell death receptor, or a nucleic acid encoding said binding member; and
(ii) a chemotherapeutic agent.

10. The method according to claim 9, wherein the binding member is the FAS antibody CH11.

11. The method according to claim 9 or claim 10 wherein the chemotherapeutic agent is 5-FU or an antifolate.

12. The use of a c-FLIP inhibitor in the preparation of a medicament for treating cancer.

13. The use of
(a) a c-FLIP inhibitor and
(b) (i) a specific binding member which binds to a cell death receptor, or a nucleic acid encoding said binding member; and/or
(ii) a chemotherapeutic agent in the preparation of a medicament for treating cancer.

14. The use according to claim 13, wherein the binding member is the FAS antibody CH11.

15. The use according to claim 13 or claim 14 wherein the chemotherapeutic agent is 5-FU or an antifolate.

16. A pharmaceutical composition for the treatment of cancer, wherein the composition comprises a c-FLIP inhibitor and a pharmaceutically acceptable excipient, diluent or carrier.

17. The pharmaceutical composition according to claim 11 wherein wherein the composition comprises a) a c-FLIP inhibitor and
(b) (i) a specific binding member which binds to a cell death receptor, or a nucleic acid encoding said binding member; and/or
(ii) a chemotherapeutic agent and
(c) a pharmaceutically acceptable excipient, diluent or carrier.

18. The pharmaceutical composition according to claim 17, wherein the binding member is the FAS antibody CH11.

19. The pharmaceutical composition according to claim 17 or claim 18 wherein the chemotherapeutic agent is 5-FU or an antifolate.

20. A kit for the treatment of cancer, said kit comprising a) a c-FLIP inhibitor and
(b) (i) a specific binding member which binds to a cell death receptor, or a nucleic acid encoding said binding member; and/or
(ii) a chemotherapeutic agent and
(c) instructions for the administration of (a) and (b) separately, sequentially or simultaneously.

21. The kit according to claim 20, wherein the binding member is the FAS antibody CH11.

22. The kit according to claim 20 or claim 21 wherein the chemotherapeutic agent is 5-FU or an antifolate.

23. The method according to any one of claims 5 to 11, the use according to any one of claims 12 to 15, the pharmaceutical composition according to any one of claims 16 to 19 or the kit according to any one of claims 20 to 22, wherein said c-FLIP inhibitor is an RNAi agent, which modulates expression of a c-FLIP gene.

24. The method according to any one of claims 5 to 11, the use according to any one of claims 12 to 15, the pharmaceutical composition according to any one of claims 16 to 19 or the kit according to any one of claims 20 to 22, wherein the c-FLIP inhibitor an RNAi agent

having nucleotide sequence AAG CAG TCT GTT CAA GGA GCA (SEQ ID NO: 1).

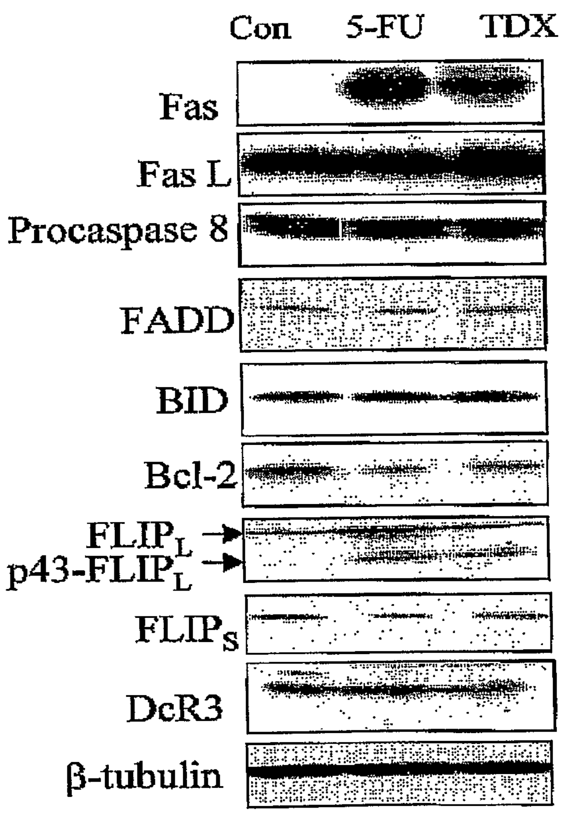

Con
5-FU
TDX
Fas
Fas L
Procaspase 8
FADD
BID
Bcl-2
FLIP$_L$
p43-FLIP$_L$
FLIP$_S$
DcR3
β-tubulin

Figure 1A

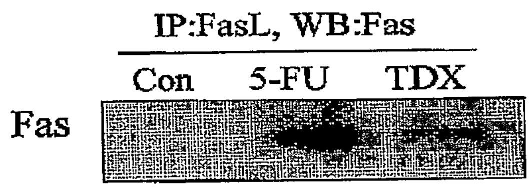


Figure 1B

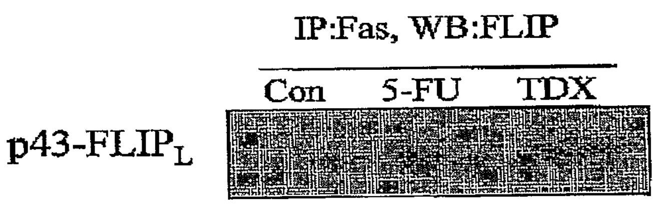


Figure 1C

Figure 2 A

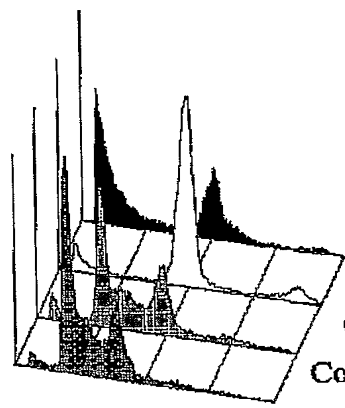


Figure 2B

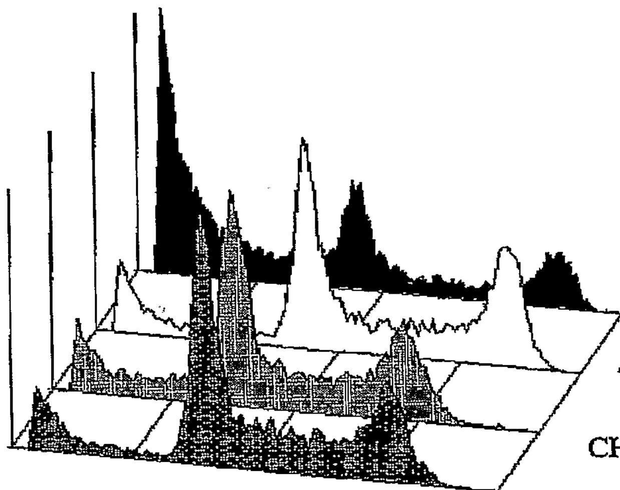


Figure 2C

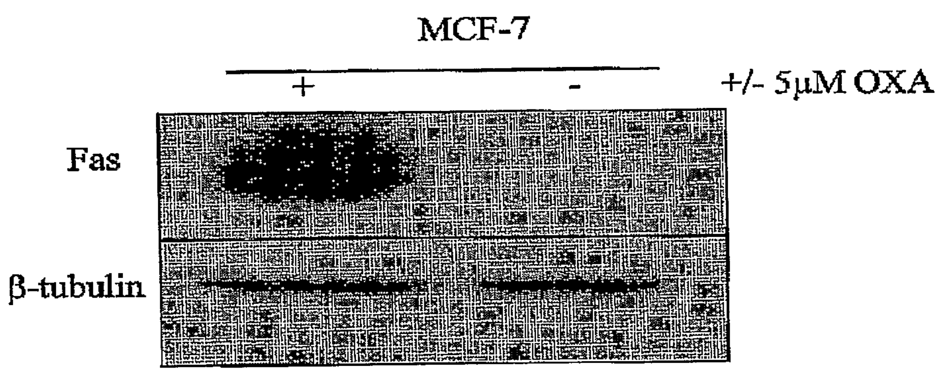
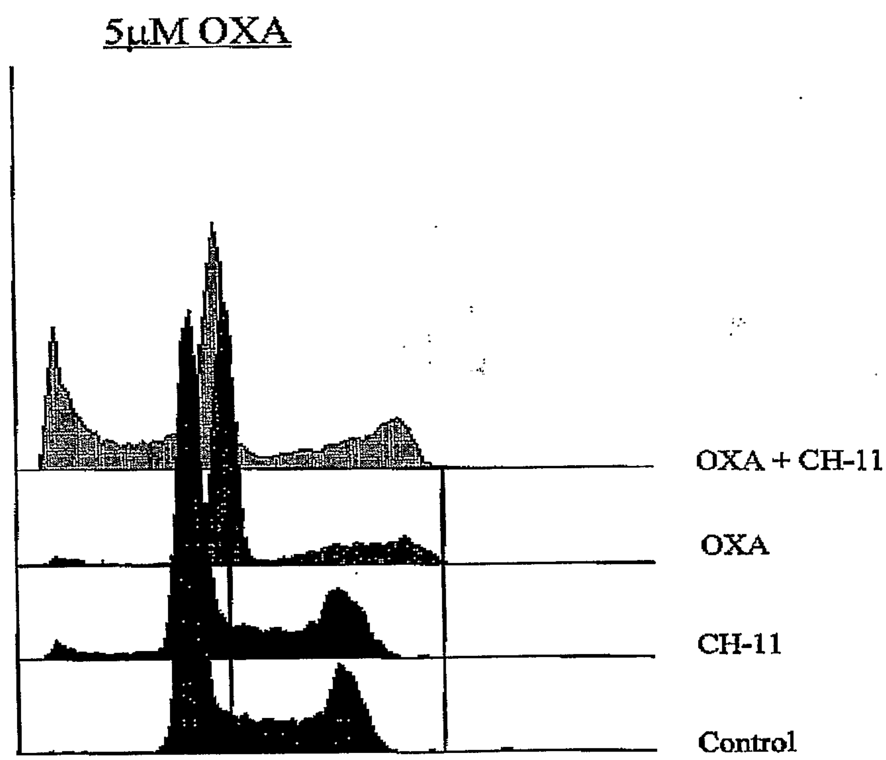


Figure 2D



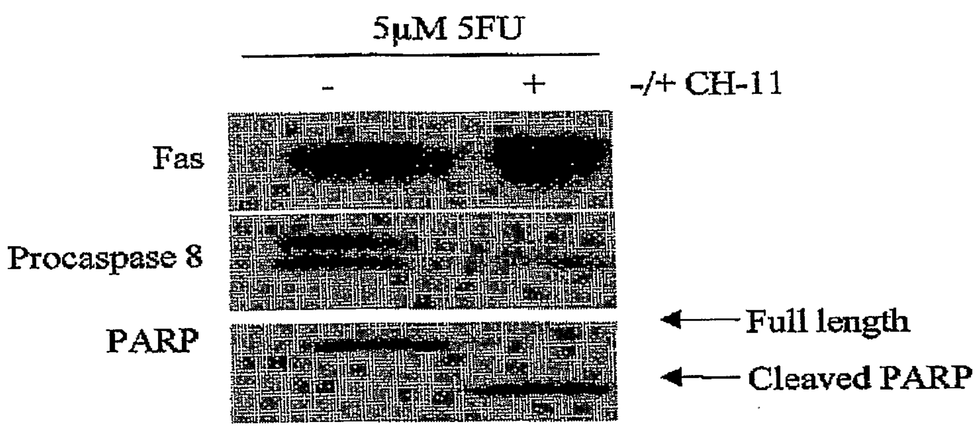


Figure 2E

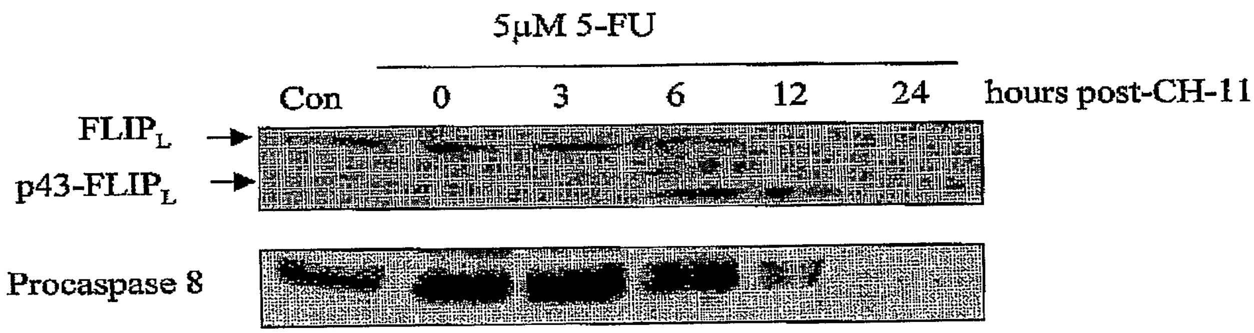

5μM 5-FU
Con
0
3
6
12
24
hours post-CH-11
FLIP$_L$
p43-FLIP$_L$
Procaspase 8

Figure 2F

Figure 3A

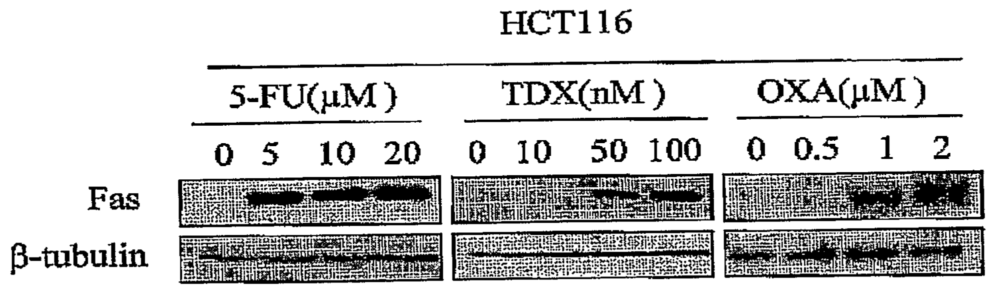
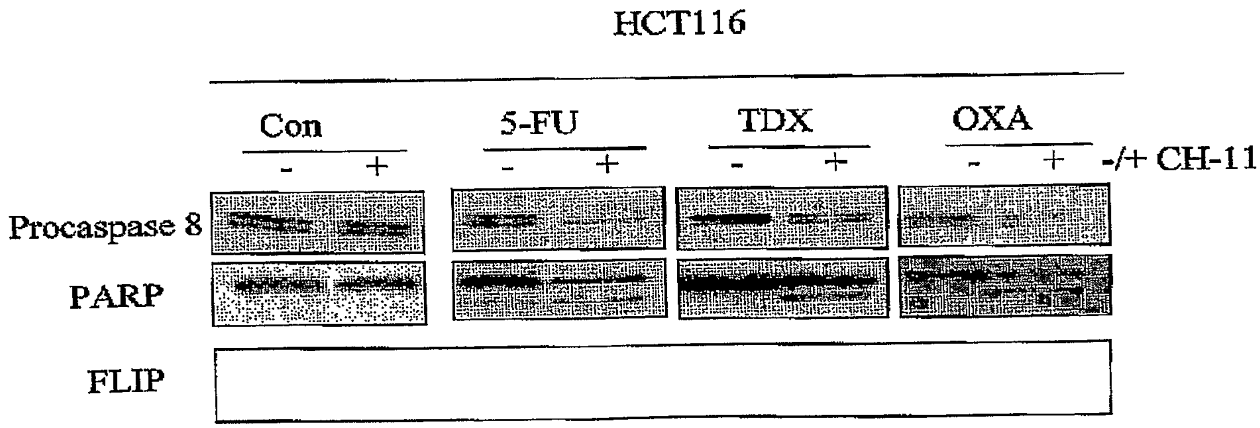


Figure 3B



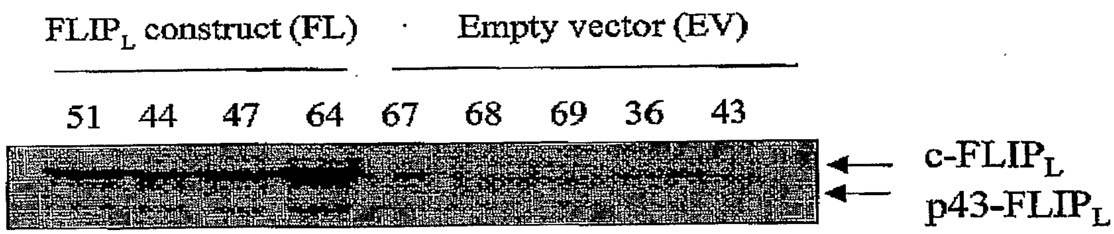


Figure 4A

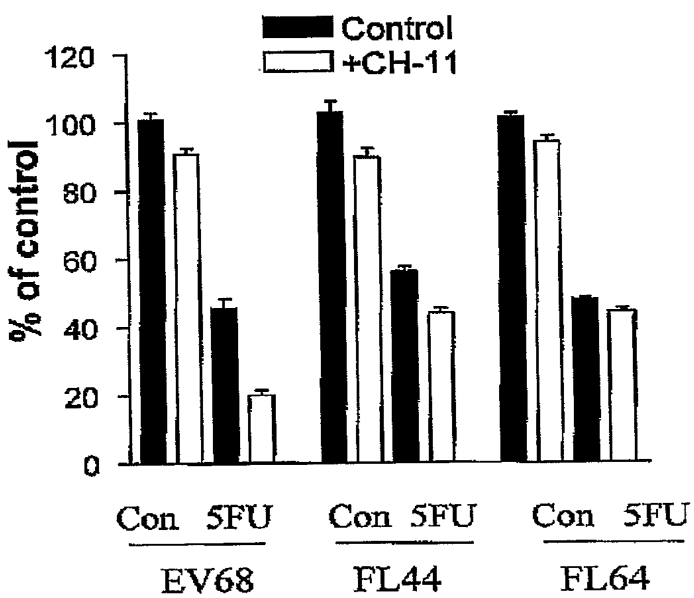


Figure 4B

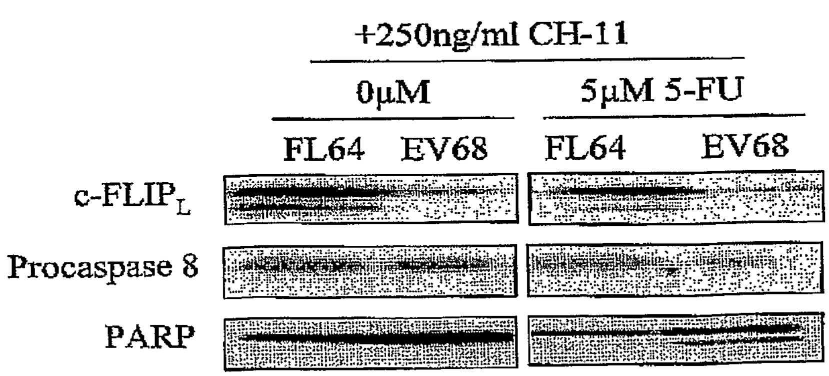


Figure 4C

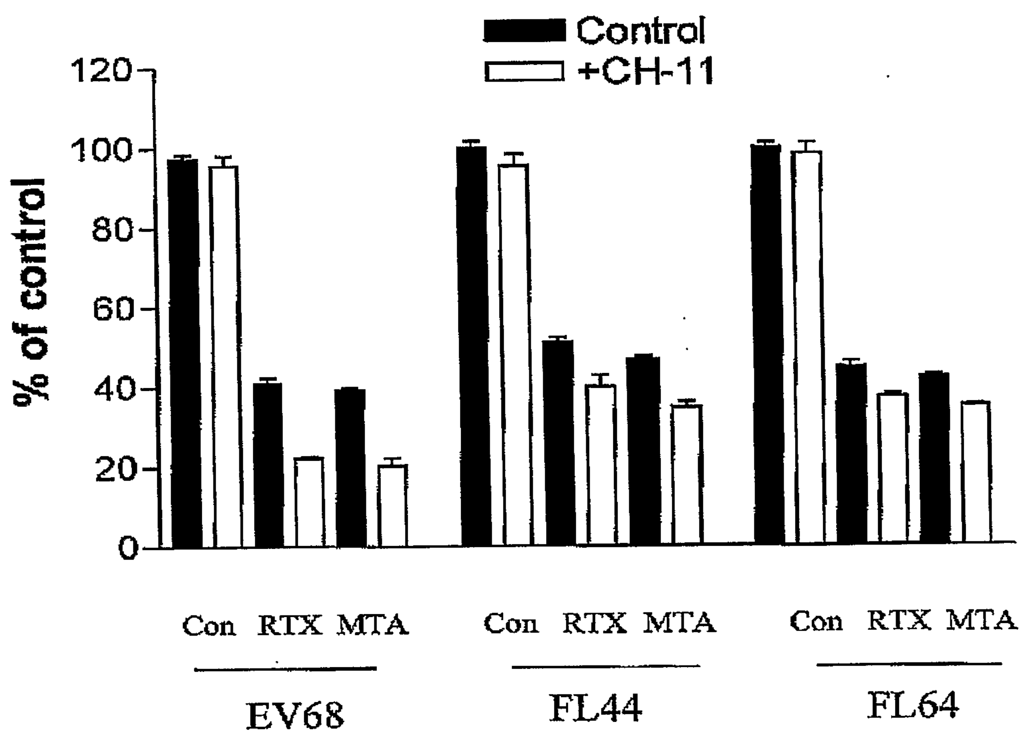
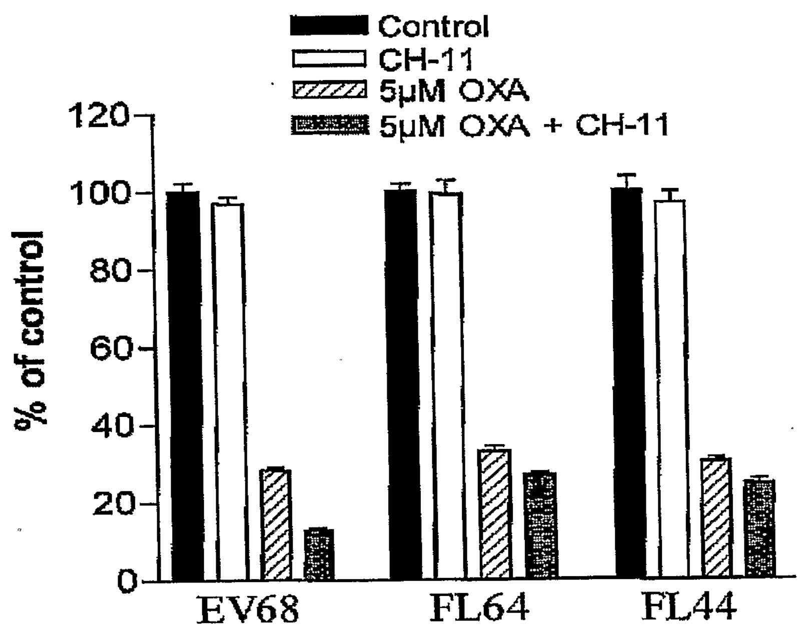

Control
+CH-11
120
100
80
60
40
20
0
% of control
Con RTX MTA
EV68
Con RTX MTA
FL44
Con RTX MTA
FL64

Figure 5A



Control
CH-11
5µM OXA
5µM OXA + CH-11
120
100
80
60
40
20
0
% of control
EV68
FL64
FL44

Figure 5B

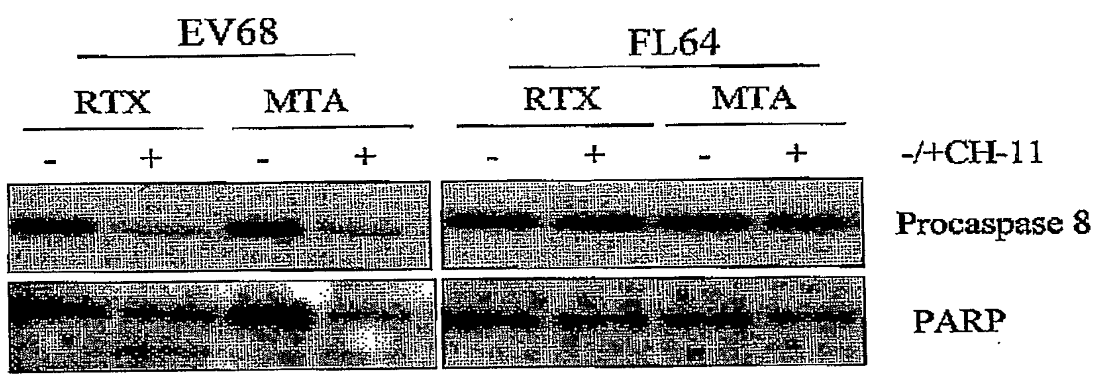


Figure 5C

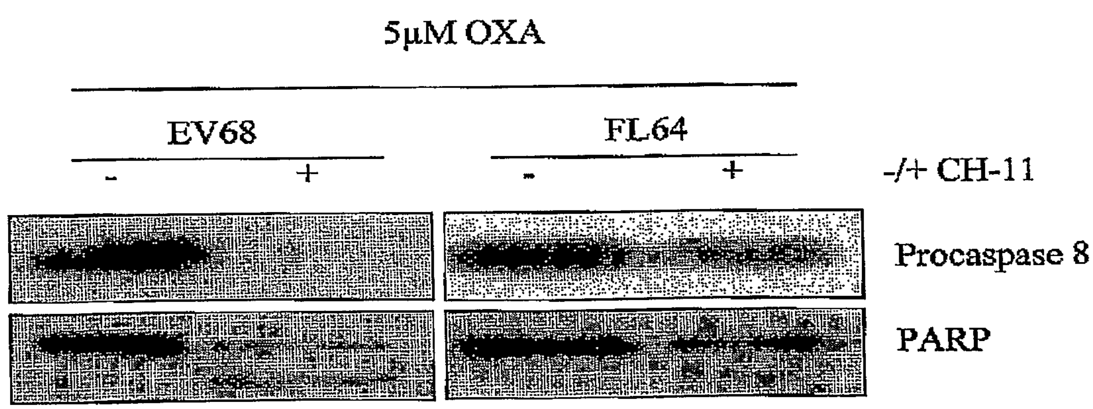


Figure 5D

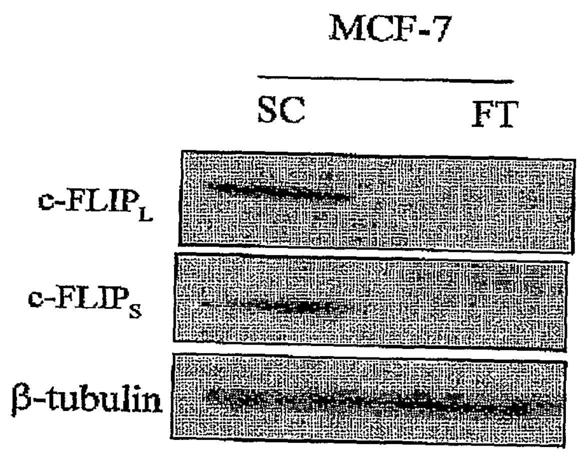


Figure 6A

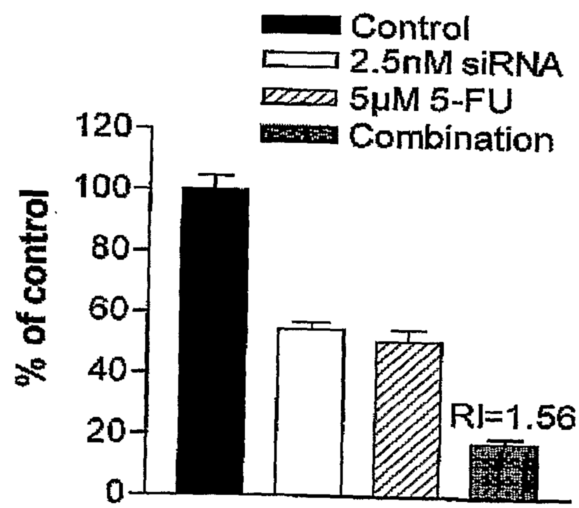


Figure 6B

Figure 6C

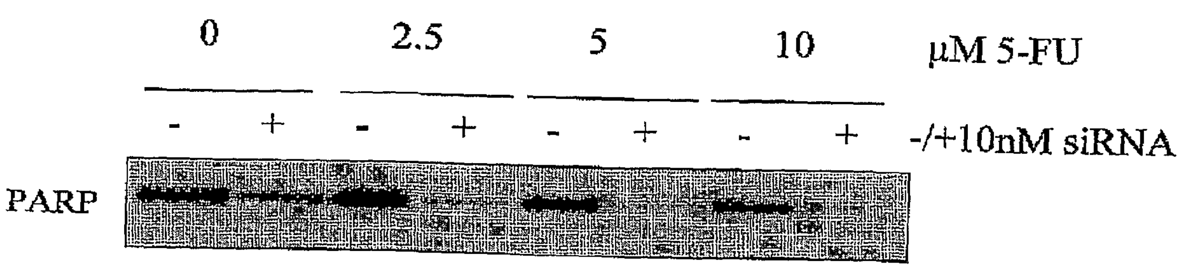


Figure 7A

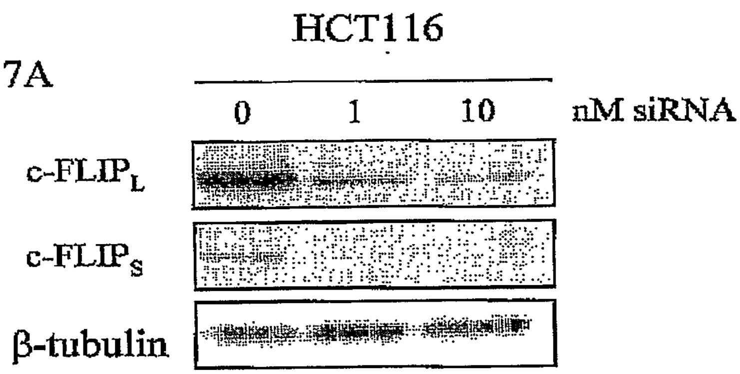


Figure 7B

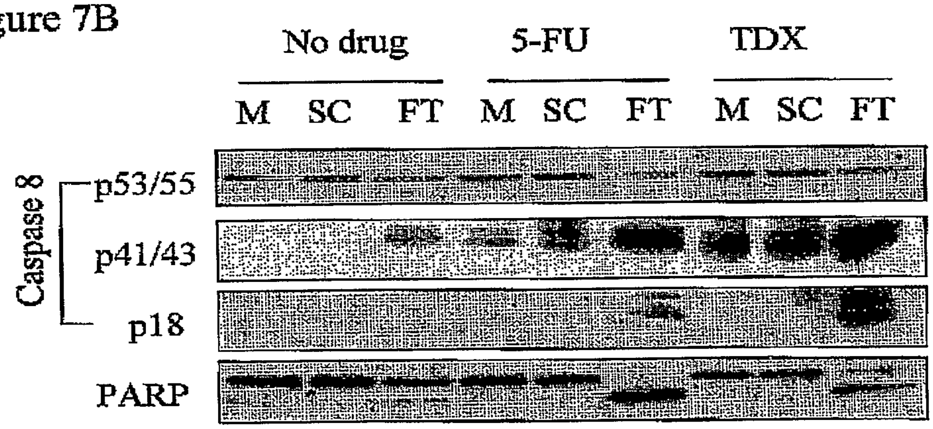
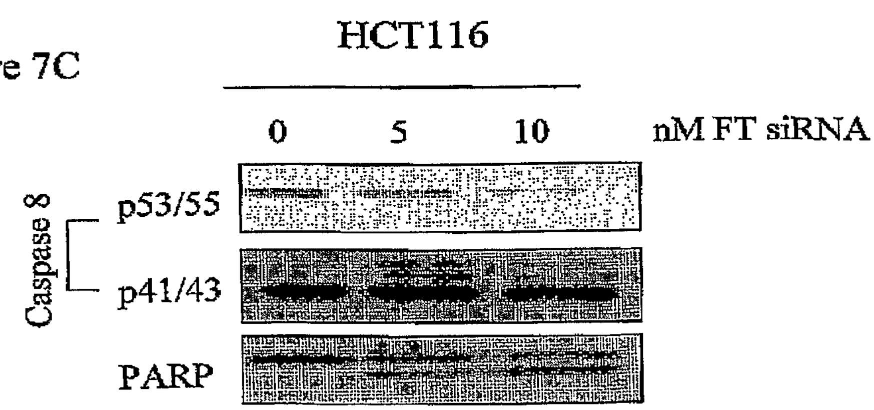
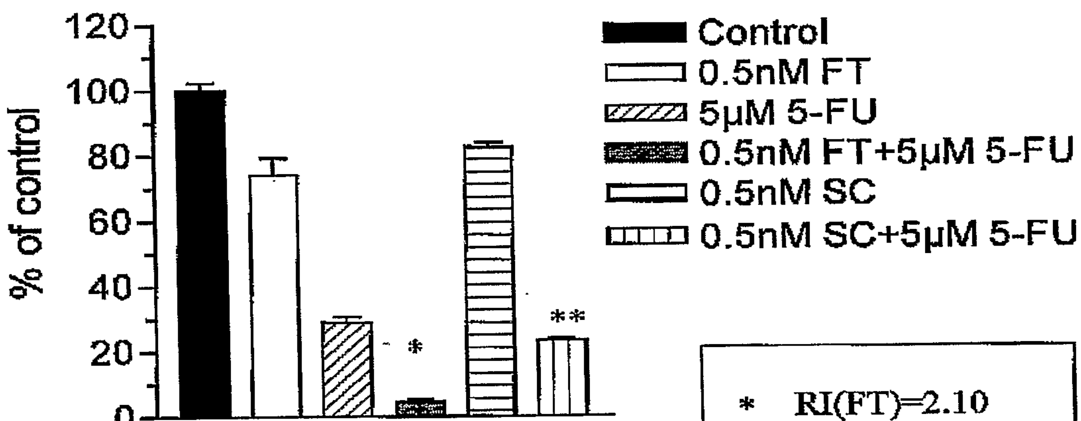


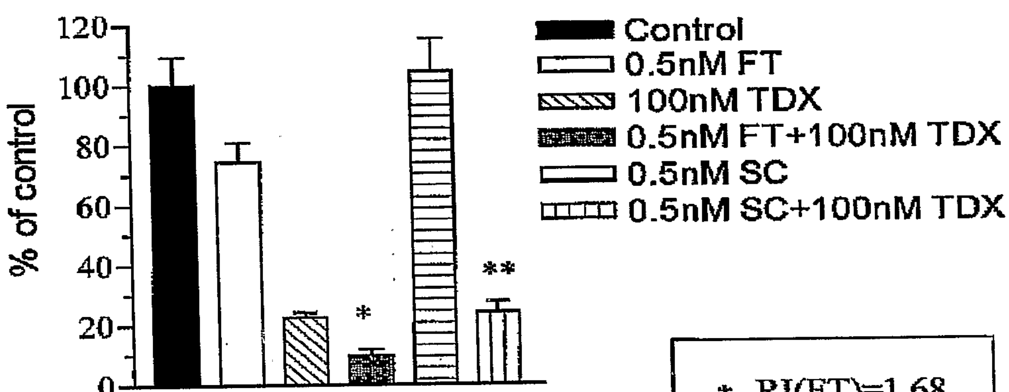
Figure 7C





*	RI(FT)=2.10
**	RI(SC)=1.00

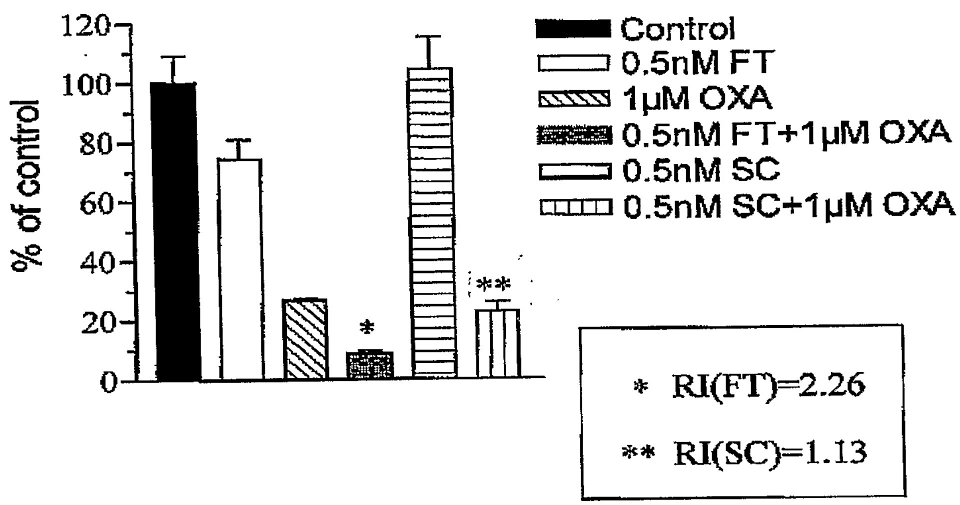
Figure 8A



* RI(FT)=1.68

** RI(SC)=0.91

Figure 8B



120
100
80
60
40
20
0
% of control
*
**
Control
0.5nM FT
1μM OXA
0.5nM FT+1μM OXA
0.5nM SC
0.5nM SC+1μM OXA
* RI(FT)=2.26
** RI(SC)=1.13

Figure 8C

PCT/GB2004/005006